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(54) Title: THE EFFECT OF TRANSGLUTAMINASE INHIBITION ON MICROFILARIAE DEVELOPMENT AND MACROFILARIAE VIABILITY

(57) Abstract

A method is described for inhibiting maturation and production of microfilariae in adult filarial nematodes. Inhibition of transglutaminase or transglutaminase mediated reactions utilizing a transglutaminase inhibitor to block the maturation and production of microfilaria by the adult organism, as well as kill the adult filarial organism. Experiments applying this method to several *Brugia* filarial infections, including *Brugia malayi*, were successful. Higher concentration of transglutaminase inhibitor prove the described filaricidal effect of the adult organism existing independent of a host or existing within a host organism.

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THE EFFECT OF TRANSGLUTAMINASE INHIBITION ON MICROFILARIAE DEVELOPMENT AND MACROFILARIAE VIABILITY

Filariasis is a group of disorders produced by infection with threadlike nematodes of the superfamily *Filarioidea*. These nematodes invade the lymphatics and subcutaneous and deep tissues of the host, even causing blindness. The viviparous female discharges microfilariae into the blood or subcutaneous tissues where they live for weeks or months until they are taken up by hematophagous arthropods. Within these arthropod vectors the microfilariae metamorphose into the infective third-stage larvae (L_3) in a period of several weeks. The L_3 larvae migrate to the mouthparts of the arthropod vector and infect a new host when the arthropod takes another blood meal. The L_3 larvae mature into adult nematodes over a period of several months and the fertilized females begin producing microfilariae, thus completing the cycle.[1]

Nearly four hundred million people worldwide suffer from chronic infections caused by filarial nematodes.[2] Filariasis is a major cause of morbidity in endemic areas of Africa, Asia, the Middle East, Pacific Islands, Central and South America, Mexico and the West Indies. Some of the more common types of filariasis are: Lymphatic filariasis produced by *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* which causes lymphatic blockade and elephantiasis; Loiasis produced by *Loa loa*, also known as African Eye Worm, characterized by transient subcutaneous (Calabar) swellings as the adult nematodes migrate under the skin, including the conjunctiva of the eye; and Onchocerciasis or River Blindness, produced by *Onchocerca volvulus*, a cutaneous filariasis causing pruritic skin rash, sclerosing lymphadenitis, subcutaneous nodules and ocular lesions often leading to blindness.[3,9] Gravid female

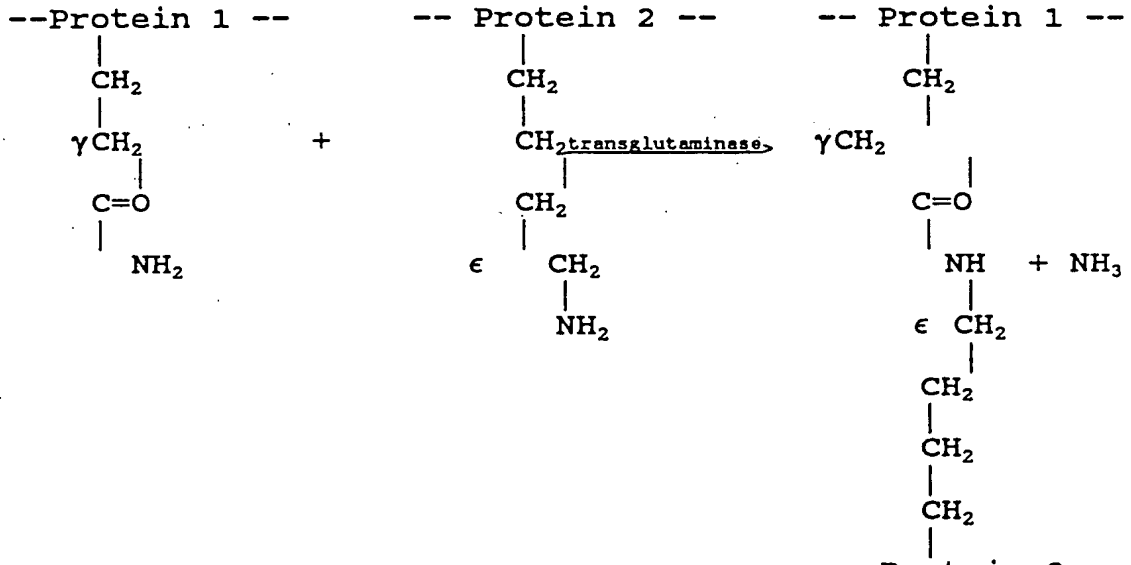
nematodes can live as long as fifteen years within a host, releasing thousands of microfilariae a day.

Available chemotherapeutic treatments are unsatisfactory as most of them are inefficacious against the mature parasites while being microfilaricidal and produce a variety of undesirable side effects. For example, the release of microfilarial antigen upon the toxic action of agents such as diethyl carbmazine (Heparigen®) or Ivermectin®, frequently induced a severe allergic response known as the Mazzotti reaction. This allergic response can sometimes be fatal, or in patients with *Onchocerca volvulus* may lead to the development of permanent eye lesions and blindness.[4] Moreover, the problem of eliminating the adult microfilaria remains, with the attendant reproduction of microfilariae by the remaining microfilaria reestablishing infective population. Additionally, the control of vectors has not proved to be sufficiently effective to interrupt transmission of the parasites, thus, at the present time, there exists no safe and reliable chemotherapeutic agent active against both the infective larvae (L_3) and adults of filarial species. Thus, a method which would both effectively eliminate both the infective larvae and adult filarial species would present a significant advancement in the control of spread of this disease, as well as improve those methods currently use in the clinical management of infected persons.

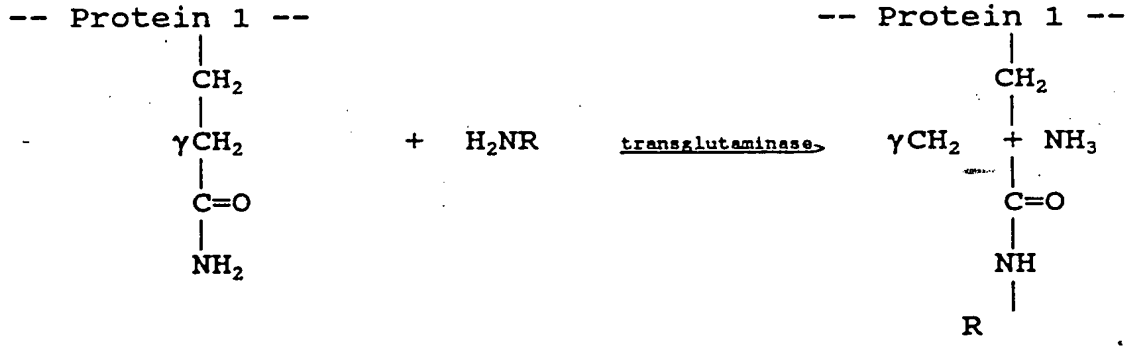
The transglutaminases (TGase; EC 2.3.2.13) are a family of Ca^{+2}-dependent enzymes that are present in a variety of cells, tissues and body fluids.[24-26] TGases catalyze highly stable post-translational covalent modification of proteins by introducing an isopeptide bond between protein-bound glutamine residues and an amine donor. The amine donor may vary from peptide-bound lysine, resulting in an $\epsilon(\gamma$-glutamyl) lysine cross link between two peptides, to a variety of other primary amine-containing compounds such as polyamines, methylamine,

and MDC. Thus, these enzymes, though structurally distinct, all catalyze the same reaction; that is cross-linking of proteins through the formation of ε(γ-glutamyl) lysine isopeptide, bonds (reaction A) as well as covalent conjugation of polyamines to protein-bound glutamine residues (reaction B).

(A)



(B)



A variety of Tgases, both intracellular and extracellular, have been identified in proteins of both mammalian systems and prokaryotes (See Table I below). These agents (TGases) have also been described as active in hair follicle formation[42], wound healing[42,43] cellular growth regulation[44,45], and differentiation[46]. These various TGases are summarized in Table

TABLE I

Proteins with TGase activity

Form	Source	MW (kDa)	Ref.
Plasma TGase (Factor XIII)		Plasma	300-320
	Platlets	150-155	19
Epidermal TGase	Keratinocytes	50-55	19
Hair-Follicle TGase	Skin	54	19
Tissue type TGase	Wide variety of tissues and cells	78-85	19, 31
Bacterial TGase	*Streptoverticillium* genus	40	21, 22
Nematodal TGase 2	Filarial adult worms	22	Fig. 1.

Additionally, a number of new transglutaminase inhibitors have been synthesized and discussed in several recently issued patents[36, 39, 40]. For example, Castelhano *et al* [40] describes certain 3,5 substituted, 4,5-dihydroisoxazoles as transglutaminase inhibitors. Inhibitors of transglutaminase include alternate substrate inhibitors, covalent inactivators and active site directed inhibitors[39].

The alternate substrate inhibitors include alkyl primary amines, such as monodansylcadaverine, and alternative acyl-donors, such as ß-phenyl propionylthiocholine. These inhibitors prevent protein crosslinking, but do not prevent post-translational modification of proteins[40]. These inhibitors have been described in the literature as suffering from the drawback of being effective only at relatively high concentrations i.e., at 10^{-3} or higher[40]. Additionally, the alkyl isocyanates transglutaminase inhibitors, as titrates of active site cysteine residues, have been described as lacking specificity for the transglutaminase[40]. Similarly, use of cystamine as a transglutaminase inhibitor has been reported as hampered by its lack of specificity for transglutaminase as

well as the high concentrations necessary to be effective[40].

A widely diverse group of conditions are described as treatable with chemical agents having transglutaminase inhibiting activity. For example, these agents have been submitted as effective in the treatment of acne, psoriasis, cataracts, immunologic disease states, Alzheimer's disease and hyaline membrane disease[36]. Additionally, dansyl cadaverine, and other amine-containing compounds such as amantadine and rimantadine, are reported to have certain antival activity[41]. The diversity of these conditions, as well as the chemical diversity of those agents described for treating them, highlight the lack of any prior appreciation for the particular utility of transglutaminase inhibitors in treating filariasis or for inhibiting/killing nematodes. Prior to this disclosure, there had been no prior teaching or suggestion that transglutaminase inhibitors of any sort would be effective against nematodes.

The conditions described in relation to the present invention do not stem from an elevation of transglutaminase activity of the host which results in a pathologic condition. Instead, the present invention relates to pathologic infective states which result from the transglutaminase activity of an infecting nematode. The particular transglutaminase inhibitor inhibits transglutaminase enzyme activity which is necessary to the nematodes' propagation and survival. The present disclosure describes the presence of a specific and novel form of TGase found in both female and male adult worms and microfilariae. This discovery was made during studies which examined the uptake incorporation of [^{3}H]putrescine and MDC into adult worms and their extracts to probe the γ-glutaminyl-containing substrates available for TGase catalysis in the nematodes under study.

The varied group of agents described as transglutaminase inhibitors in the literature may be broadly characterized as either reversible or irreversible transglutaminase inhibitors. For example, monodansyl cadaverine, regarded as a reversible transglutaminase inhibitor, cystamine and iodoacetamide are examples of irreversible transglutaminase inhibitors, as therapeutic agent, those transglutaminase inhibitors which are reversible would be preferred. Generally, those transglutaminase inhibitors which act by competing for transglutaminase enzyme substrate, and which further include a primary amine group are classified as reversible transglutaminase inhibitors.

Involvement of TGase-catalyzed reactions in fertilization and embryogenesis has been studied by several investigators. For example, *Cariello et al*[37] observed an increase in TGAse-catalyzed incorporation of [^{3}H]putrescine into sea urchin eggs, 20 min after fertilization; incorporation could be completely blocked by the TGase substrate inhibitor MDC. Also, the presence of high intracellular TGase activity during early stages of development was confirmed by demonstration of ϵ(γ-glutamyl) lysine bonds[30]. Similarly, *Battaglia et el*[38] observed that inhibition of TGase by putrescine and MDC resulted in disorganized and enlarged fertilization envelope formation and concluded that egg surface TGase catalyzes covalent cross-linking of proteins in sea urchin during assembly of the fertilization envelope.

More recently, *Cariello* and coworkers[30] have reported the *in vivo* TGase-catalyzed post-translational modification of proteins in sea urchin during early developmental stages. Involvement of TGase during preimplantation mouse embryogenesis was studied by *Maccioni* and *Arechaga*[31]. For example, a significant increase in TGase activity was observed during the transition from 2-cell embryos to 8-cell morulae or blastocysts. In the free living nematode *Caenorhabditis*

elegans, and in the gastrointestinal parasite *Ascaris lumbricoides*, the external cortex, containing the epicuticle, has been shown to possess the covalently cross-linked, highly insoluble protein termed 'cuticulin'[35,36]. More recently, *Selkirk* and co-workers[34] have observed the presence of such insoluble structures in adult worms of *B. malayi* and *B. pahangi*. Interestingly, the major component protein in 'cuticulin' has been shown to be collagen.[32] Substantial synthesis of collagen in filarial parasites during the fourth stage larvae (L_4) - adult moult has been observed.[34] Whether these larval stages express pTGase would be of interest in the development of methods which effect filarial maturation, growth and regeneration.

The inventors herein postulate that TGase in filarial parasites is responsible for cross-linking of host/parasite proteins, and that further, this pTGase-catalyzed post-translational modification of proteins may be important in the assembly of egg shell or sheath. Determination of whether TGase is also responsible for cross-linking of host/parasite proteins in filarial parasites, and if these pTGase-catalyzed post-translational modifications are important to the assembly of egg shell or sheath filarial formation, would be valuable in the development of effective methods for the control and eradication of filarial nematodes and the filarial diseases they cause.

In mammalian hosts, the TGase gene has been shown to be under the direct control of retinoids[35]. Retinoic acid seems to interact with nuclear retinoic acid receptor in the target cells, leading to the transcriptional activation of the TGase gene. Studies on delineation of the host/parasite factors that regulate the expression of the pTGase gene in filarial parasites would help pinpoint the possible sites of attack for novel filarial parasite (i.e., transglutaminase) inhibitors and also help identify the key pathways involved in growth and

differentiation of filarial parasites. Since these parasites lack visual function, the retinoids present in them are postulated to be perhaps utilized solely to support their growth, differentiation and reproduction. Thus, characterization methods of inhibiting expression of the TGase gene would present a valuable technique in regulating filarial growth and maturation.

A discovery important in the management of filarial parasites is reported herein. The present inventors describe a unique transglutaminase in filarial organisms which is sensitive to a variety of hemal substances generally described and characterized herein as transglutaminase inhibitors. The transglutaminase inhibitors may be further characterized as reversible transglutaminase inhibitors or irreversible transglutaminase inhibitors. Those reversible transglutaminase inhibitors include, for example, monodansyl cadaverine, putrescine, methyl amine and histamine. Irreversible transglutaminase inhibitors include, by way of example, cystamine and iodoacetamide. The filarial transglutaminase, for which specific transglutaminase inhibitors have been herein characterized, has been found to be critically important in the formation and development of microfilariae *in utero* in adult female filarial nematodes. Specifically, inhibition of this particular transglutaminase in female filarial nematodes with the described methods is reported herein to halt the normal development of microfilariae.

Transglutaminase mediated activities are also shown to be critical to the function and survival of both male and female adult filarial nematodes, as treatment of these organisms with a sufficient dose of an inhibitor of the transglutaminase has been found to be filaricidal.

By determining the presence and importance of substrates in

filarial nematodes for TGase, and the role of TGase-mediated reactions in filarial development and growth, the inventors provide methods for inhibiting microfilarial production and maturation, as well as killing adult filarial organisms, through the use of any agent which inhibits the filarial transglutaminase characterized by the inventor.

By way of example, particularly preferred transglutaminase inhibitors include monodansyl cadaverine, dansylcadaverine, putrescine, histamine, methyl amine, cystamine, iodoacetamide dansyl cadaverine, amantadines, rimantidine, ß-phenyl propionylthiocholine, and a 3,5 substituted 4,5-dihydroisoxazole. A transglutaminase inhibitor, as that term is used in the present disclosure, is defined as an agent which is capable of inhibiting the enzymatic activity of a filarial transglutaminase enzyme or a physiological function dependent or mediated by a filarial transglutaminase enzyme action.

A preferred transglutaminase inhibitor, monodansyl cadaverine, is demonstrated to have extremely potent activity in this regard. However, any one or combination of transglutaminase inhibitors are reasonably expected to be suitable for use in the claimed invention. For example, transglutaminase inhibitors such as dansyl cadaverine, putrescine, histamine, methyl amine, cystamine, iodoacetamide, ß-phenyl propionylthiocholine, amantadine, rimantadine, or any of a variety of 3,5 substituted, 4,5-dihydroisoxazoles may be used as the transglutaminase inhibitor of choice. Administered parenterally, transdermally or possibly internally, treatment with a transglutaminase inhibitor, such as monodansyl cadaverine, offers a means of treatment of filariasis in humans while avoiding the side effects of toxicity or allergic response typical of other forms of drug therapy. The present disclosure provides specific methods whereby this inventive discovery may be used to control human

filarial parasites.

The present invention provides both an *in vivo* and *in vitro* method for blocking production and maturation of microfilariae by a female filarial nematode. Most particularly, this method comprises administering a transglutaminase inhibiting agent to the female filarial nematode, most particularly in an amount sufficient to halt or inhibit microfilarial development. The transglutaminase inhibiting agent as used in the present invention is also generally referred to as a transglutaminase inhibitor which is capable of inhibiting transglutaminase enzyme activity *in vivo* or *in vitro*. This method, for example, inhibits production and maturation of microfilariae in an adult female filarial nematode, such as in *Brugia malayi*, *Brugia pahangi* or *Brugia patei*. Of course, this method would also be applicable in the treatment of other filarial nematodes such as *Brugia timori*, *Wuchereria bancrofti*, *Loa loa or Onchocerca volvulus*.

In one embodiment, inhibiting production and maturation of microfilariae by a female filarial nematode of *Brugia malayi*, *Brugia pahangi or Brugia patei* is accomplished by an *in vitro* method comprising treating said nematode with a transglutaminase inhibiting agent in an amount sufficient to inhibit said production and maturation of microfilariae.

As a method for inhibiting the production of microfilariae of *Brugia malayi* and other filarial nematodes *in vivo*, for example in a mammalian host, the present invention comprises administrating a pharmacologically acceptable concentration of a transglutaminase inhibitor to the mammalian host. Most preferably, the transglutaminase inhibitor may be selected from the group consisting of dansyl cadaverine, monodansyl

cadaverine, putrescine, histamine, methylamine, iodoacetamide, cystamine, 3,5 substituted, 4,5-dihydroisoxazoles, ß-phenyl propionylthiocholine, amantadine and rimantadine. The particular transglutaminase inhibitor of the present invention may comprise a reversible transglutaminase inhibitor or a irreversible transglutaminase inhibitor. The reversible transglutaminase inhibitors, for example, monodansyl cadaverine, are most particularly preferred in the presently disclosed invention. Monodansyl cadaverine is most particularly preferred as the transglutaminase inhibitor of choice for use in the presently described methods in the control of nematodes and the disease they cause.

A transglutaminase inhibotor for use in killing macrofilariae (i.e., adult filarial nematodes) in a mammalian host, is also provided. The transglutaminase inhibitor as used to kill adult nematodes by administering a filaricidal concentration of the transglutaminase inhibitor to the mammalian host. In its most preferred application, the transglutaminase inhibitors disclosed herein are used in the treatment of a rodent or a human mammalian host. Most preferably, the host is a human. The administration of the transglutaminase inhibitor as described would also inhibit the production of microfilariae in the host. Thus, a transglutaminase inhibitor for use in the treatment of filariasis is provided.

The transglutaminase inhibitors utilized in this invention include any agent which elicits a reduction in transglutaminase enzyme activity or of a transglutaminase-enzyme or regulated physiological response. By way of example, monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] comprises the most preferred transglutaminase inhibitor of the present invention, but other transglutaminase inhibitors or pseudo-substrates of transglutaminase, are understood to be included in the

category termed "transglutaminase inhibitors" as used in the present disclosure.

Additionally, the category of transglutaminase inhibitors includes those agents which demonstrate an ability to inhibit filarial maturation or inhibit microfilarial production in the female filarial organism. In a most preferred embodiment, the concentration of transglutaminase inhibitor used to inhibit production of microfilariae in an adult female filarial nematode is about a 200 μM concentration. However, it should be possible to employ lesser or greater concentrations of various other agents which possess the described specific transglutaminase-inhibiting activity, for example when an inhibitor other than monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] is utilized.

In another embodiment, a method for killing an adult filarial nematode (i.e., a macrofilariae) is provided. This method comprises treating the filarial nematode with a filaricidal concentration of a transglutaminase inhibitor. A filaricidal concentration of a transglutaminase inhibitor is generally a concentration greater than that merely inhibitory of microfilariae production. For example, a filaricidal concentration of monodansyl cadaverine effective for killing an adult filarial nematode is greater than about a 300 μM concentration. This method would pertain to all the filarial infections previously listed.

In a more preferred embodiment, the present invention involves a method for inhibiting production of microfilariae by an adult female filarial nematode existing within a mammalian host. This method comprises treating the mammalian host with a transglutaminase inhibitor in an amount sufficient to inhibit the transglutaminase activity of an adult female filarial nematode or sufficient to inhibit the development of microfilaria which may be within the female filarial nematode.

Another preferred embodiment provides a method for inhibiting production of microfilariae in an adult female *Brugia malayi* nematode existing within a mammalian host. This method comprises treating the mammalian host with a transglutaminase inhibitor in an amount sufficient to inhibit the transglutaminase activity in the adult female *Brugia malayi* nematode.

The transglutaminase inhibitor used to inhibit production of microfilariae in an adult female filarial nematode existing within a mammalian host, including the *Brugia malayi* nematode, is most preferably monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] and most preferably in an about 200 μM concentration in the host. However, this choice of transglutaminase inhibitor and concentration may be subject to the provisions previously stated.

In another embodiment, this invention provides a method for treating filariasis in a mammalian host comprising administering a transglutaminase inhibitor to the host in an amount sufficient to kill the adult filarial nematodes producing the filarial infection. *Onchocerciasis* and *Loiasis* would be among the filarial infections to be treated by this method. All of the previously listed transglutaminase inhibitors are expected to be effective in this regard. The most particularly preferred transglutaminase inhibitor for use is monodansyl cadaverine.

In another preferred embodiment, a method for treating lymphatic filariasis in a mammalian host is provided. A most preferred embodiment of this method comprises administering to the host a transglutaminase inhibitor in an amount sufficient to kill the adult filarial nematodes producing the lymphatic filariasis. This method, which most preferably may be utilized for treating a specific type of lymphatic filariasis, comprises administering to a mammalian host a transglutaminase

inhibitor in an amount sufficient to kill adult *Brugia malayi* nematodes producing the particular lymphatic filariasis. This method may be used in treating lymphatic filariasis produced, for example, by *Brugia timori, Brugia pahangi, Brugia patei* or *Wuchereria bancrofti*.

The transglutaminase inhibitor administered to a mammalian host to treat filariasis or lymphatic filariasis is most preferably monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide]. However, any transglutaminase inhibitor having filaricidal activity may be employed in the claimed method, and includes any of those transglutaminase inhibitors reported in the art, [36, 39-43] which references are specifically incorporated herein by reference for this purpose, and are further subject to the previously stated provisions.

The mode of administration of the transglutaminase inhibitor to a mammalian host may be parenteral, for example intravascular or more specifically intravenous. The transglutaminase inhibitor might even be supplied on a continuous dose basis transdermally *via* a skin patch or given internally. Depending upon the clearance rate of the transglutaminase inhibitor within the mammalian host, daily, weekly or perhaps even a monthly dose regimen could be followed in treating the filariasis.

The mammalian host to which the transglutaminase inhibitor is administered for treatment of filariasis or for blocking maturation and production of microfilariae in an adult filarial nematode within said host, may be a rodent, a dog, cat or primate such as a monkey or a human.

In another preferred embodiment, this invention provides a pharmaceutical formulation for treating filariasis in a mammalian host comprising administering to the mammalian host

a filaricidal concentration of a transglutaminase inhibitor in pharmaceutically acceptable excipient. The filaricidal concentration of the particular transglutaminase inhibitor selected should be capable of killing the particular adult filarial nematodes in the mammalian host which are producing the filariasis condition in the mammal but which is not toxic to the host itself. Filarial infections within a mammalian host such as: *Onchocerca volvulus, Loa loa, Wuchereria bancrofti, Brugia timori, Brugia pahangi* or *Brugia patei*, may all be treated with such a pharmaceutical formulation.

In yet another preferred embodiment, a pharmaceutical formulation is provided for treating lymphatic filariasis specifically produced by *Brugia malayi* in a mammalian host comprising a transglutaminase inhibitor in an amount sufficient to kill adult *Brugia malayi* nematodes producing the lymphatic filariasis, in combination with a pharmaceutically acceptable excipient. Again, the dose or concentration of the transglutaminase inhibitor must be such as to not elicit a toxic effect on the host, and comprise a pharmacologically acceptable concentration to the host selected. However, the concentration of the transglutaminase inhibitor must be sufficient to elicit a filariacidal effect on the infecting nematodes.

In the last two preferred embodiments, monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide], is the transglutaminase inhibitor of choice. However, other transglutaminase inhibitors or pseudo-substrates may be used in the pharmaceutical formulation as previously discussed in the provisions for transglutaminase inhibitors. It is also understood that the mammalian hosts to which the pharmaceutical formulations are applied are the same as previously mentioned: a rodent, dog, cat or primate such as a monkey or a human.

These and other aspects of the present invention will become more readily apparent when viewed in the context of the description of specific embodiments in the examples set forth below. However, neither the summary, the description or the examples are intended to limit the scope of the claims unless expressly stated therein.

Abbreviations used herein include:

CHAPS	3-[(3-cholamidopropyl) dimethylamino]-1-propane sulfonate
CO_2	carbon dioxide
CUB-7401	monoclonal antibody against guinea pig liver tissue transglutaminase
DDC	dimethyl-dansyl cadaverine
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol (2-aminoethyl ether) tetraacetic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GACT	γ glutaminyl cyclotransfirase
IgG	immunoglobulin G
IP	intraperitoneally
IV	intravenously
Kg	kilogram
L_3	infective third-stage larvae
L^4	fourth stage larvae
μl	microliter
μM	micromolar
MDC	monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide]
Mf	microfilariae
mg	milligram
ml	milliliter
mM	millimolar
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pTGase	transglutaminase enzyme from parasites
SDS	sodium dodecylsulfate
TGase	transglutaminase enzyme
UV	ultraviolet light

Figure 1A is a dose response curve showing the effect of increasing concentration of monodansyl cadaverine (DC) (a transglutaminase inhibitor) on the release of microfilariae by *Brugia malayi* adult female nematodes. The results shown are the average number of microfilariae released by two female nematodes from four independent experiments. The standard deviation was less than ten per cent. INSET: The average number of microfilariae released into the spent medium by two *Brugia malayi* female nematodes over one to four days of incubation in the absence of 50 μM monodansyl cadaverine (MDC) (●) or presence of 50 μM monodansyl cadaverine (MDC) (O).

Figure 1B demonstrates the morphology of the developing embryos in the uterus of the *Brugia malayi* female nematode as seen under phase-contrast microscopy (X400) after 3 days of incubation in RPMI 1640 medium.

Figure 1C demonstrates the morphology of the developing embryos in the uterus of the *Brugia malayi* female nematode as seen under phase-contrast microscopy (X400) after three days of incubation in RPMI 1640 medium containing 200 μM monodansyl cadaverine (MDC). Embryos are lesser in number and are undifferentiated.

Figure 2A demonstrates the detection of transglutaminase by immunoblotting in soluble and insoluble extracts of adult female *Brugia malayi* and the absence of the enzyme in the male adult nematodes of same species. Immunodetection of transglutaminase on nitrocellulose paper was performed using anti-tissue transglutaminase monoclonal antibody (CUB 7401) and alkaline-phosphatase-coupled goat antibody to mouse immunoglobulin G as second antibody. Lane 1: Molecular weight markers; Lane 2: purified guinea pig liver transglutaminase (1 μg); Lane 3: extracts from male nematodes; Lane 4: soluble extracts from female nematodes; Lane 5: insoluble extracts from female nematodes.

Figures 2B shows the immunoprecipitation of the protein extracts from adult male and female *Brugia malayi* nematodes. SDS-gel electrophoresis of CUB 7401 immunoprecipitated soluble and insoluble extracts of the male and female adult nematodes were detected by fluorography. Lane 1: immunoprecipitate of soluble extracts of male nematodes; Lane 2: immunoprecipitate of soluble extracts of female nematodes; Lane 3: immunoprecipitate of insoluble extracts of female nematodes.

Figure 2C indicates the total protein profiles of the adult *Brugia malayi* nematodes. SDS-gel electrophoresis fractions of soluble and insoluble protein extracts of adult female and male nematodes were subjected to continuous PAGE fractionation followed by Coomassie blue protein staining. Lane 1: purified guinea pig liver transglutaminase (5 μg); Lane 2: soluble fraction of adult male nematodes; Lane 3: soluble fraction of adult female nematodes; Lane 4: insoluble fraction of female nematodes; Lane 5: molecular weight markers.

Figure 3A and 3B demonstrate indirect immunofluorescence labeling of the *Brugia malayi* adult female nematode using CUB 7401 antibody. Figure 3A shows the immunofluorescence under a Leitz phase-contrast microscope and Figure 3B shows the immunofluorescence under a fluorescence microscope (40 X objective). In control experiments, normal mouse or rabbit sera or second antibody alone failed to produce immunofluorescence (data not shown).

Figure 4A and 4B represent detection of substrate proteins used by exogenous guinea pig liver transglutaminase and endogenous transglutaminase in *Brugia malayi* adult male and female nematodes. Figure 4A shows that the number and the amount of substrate proteins is much higher in the female nematodes than in the male nematodes as visualized by SDS polyacrylamide-gel electrophoresis under UV light. Lane 1: Molecular weight markers; Lane 2: Guinea pig liver

transglutaminase, extracts of adult male nematodes, 400 μM monodansyl cadaverine (MDC) and CaCl; Lane 3: Guinea pig liver transglutaminase, extracts of female nematodes, 400 μM monodansyl cadaverine (MDC) and $CaCl_2$ Lane 4: Guinea pig liver transglutaminase, extracts of male nematodes, 400 μM monodansyl cadaverine (MDC) and EGTA; Lane 5: Guinea pig liver transglutaminase, extracts of female nematodes, 400 μM MDC and EGTA; Lane 6: Endogenous transglutaminase from adult female nematodes with 200 micromolar monodansyl cadaverine (MDC); Lane 7: untreated adult female nematode extracts as the control. Figure 4B is the Coomassie blue stained gel of the protein samples shown in Figure 4A.

Figure 5 shows the isolation and morphological properties of embryonic sheaths. Phase-contrasts (A) and scanning-electron microscopic (B) appearance of sheaths isolated by detergent treatment from *in utero* developing embryos.

Figure 6 indicates immunofluorescence labeling of embryos by CUB 7401 antibody. Uterine contents from female worms were obtained as described *infra*. Dried smears were fixed with methanol at -20°C and permeabilized with 0.2% CHAPS detergent in phosphate-buffered saline. Permeabilized specimens were stained and examined under phase-contrast (A) or fluorescence microscope (B), as described for Fig. 3.

Figure 7 demonstrates *in situ* labeling of embryos with MDC. Uterine contents from MDC-treated female worms were obtained as described *infra*. Unbound MDC from the embryos was eluted by extensive and repeated washing in acid alcohol, followed by extensive washing in Tris-buffer saline to neutralize the pH. The smears were mounted in 90% glycerol in PBS and observed under phase-contrast (A) and fluorescence microscope (B), using a blue excitation radiation filter.

Figure 8 shows the scanning electron microscopic appearance

of *in utero* developing embryos, obtained from *B. malayi* female worms incubated for 72 h culture in presence of medium alone (A), or medium containing 200 μM MDC (B).

Figure 9 reflects the reversibility of MDC-induced suppression of mf release in *B. malayi* female worms. Female worms were incubated in the presence of medium containing 200 μM MDC for 24 h (-0-), 48 h (-▒-), 72 h (-□-), or 96 h (-●-). At the end of each incubation period, worms were removed and washed and two female worms were incubated further in each 1 ml of drug-free medium. At indicated time points, the number of Mf released into the spent medium were counted as described under legend of Fig. 1. A MF released by untreated control worms are also counted (-●-). The results represent an average number of Mf released by two female worms from two independent experiments in duplicate. Standard deviation from the mean was less than 10%.

Figure 10 indicates covalent post-translational modification of *B. malayi* female worm proteins [^{35}S]Methionine/cysteine-labeled female worms were incubated at 37°C in presence of serum-containing medium. At indicated time intervals, three female worms each were used for fractionation of proteins on SDS-PAGE as described *infra*. The labeled protein bands were detected by autoradiography. The first lane on the left indicates the mobility of standard molecular weight markers.

Figure 11 represents localization of ^{35}S-labeled proteins in *utero* developing embryos of *B. malayi* female worms. Labeled parasites were incubated in presence of medium alone (A), or medium containing 200 μM MDC (B). After 24 h of incubation, the uterine contents from untreated and treated worms were obtained and processed for autoradiography as descried *infra*.

Figure 12 indicates the chemical structure of monodansyl cadaverine (MDC) and its inactive analogue, dimethyldansyl-cadaverine (DDC)and various other primary amines which serve as substrate inhibitors for TGase.

Figure 13 - Elution profile of sample without (A) and after (B) γ-glutaminyl cyclotransferase (GACT) treatment on a C18 HPLC column.

The present disclosure describes a method for inhibiting or blocking the production and maturation of microfilariae by female filarial nematodes by inhibiting transglutaminase activity. Also disclosed is a method for killing adult filarial nematodes. The described method for killing adult filarial nematodes comprises administering to or exposing the filarial nematode to a filaricidal concentration of a transglutaminase inhibitor. The particular transglutaminase inhibitors found to provide the best mode of practicing the invention at this time is monodansyl cadaverine. However, any of the transglutaminase inhibitors referenced herein may be employed in accordance with the present invention.

The inventors have shown that a 22 kD transglutaminase found only in the adult female filarial nematodes, is essential for the differentiation of the early embryonic stages to the mature microfilariae *in utero*. Through the use of transglutaminase inhibiting agents such as monodansyl cadaverine, histamine, putrescine, methyl amine, cystamine or iodoacetamide, or any combination thereof, for example, the production of filarial nematodes may be limited.

Monodansyl cadaverine (MDC) was found to be the most effective transglutaminase inhibitor among those tested for blocking the maturation and production of microfilariae *in vitro* by three different *Brugia* species which cause lymphatic filariasis, *Brugia malayi, Brugia pahangi and Brugia patei*.

MDC inhibition proceeded in a dose-dependent manner. Of course, monodansyl cadaverine is not the only transglutaminase inhibitor or pseudo-substrate that can be utilized in this capacity. All such pseudo-substrates are considered to fall within the scope of this invention. Also, there is no reason to limit the application of monodansyl cadaverine only to these aforementioned *Brugia* infections, as it should also be applicable to control of other filarial infections such as lymphatic filariasis caused by *Wuchereria bancrofti and Brugia timori, Loiasis and Onchocerciasis* for example. Therefore any transglutaminase inhibiting agent which inhibit filarial maturation and production are included among those transglutaminase inhibitors of the present invention.

Experiments with intraperitoneally transplanted *Brugia malayi* adult nematodes in nude mice indicated that treatment of a mammalian host intravenously with a transglutaminase inhibitor, such as monodansyl cadaverine, could cause the death of the filarial nematodes while leaving the host free of toxic symptoms. The dose of transglutaminase inhibitor, MDC, given *in vivo*, was about 5 times as concentrated as was necessary to inhibit maturation and production of microfilariae *in utero* in adult female filarial nematodes.

Toxicity studies with monodansyl cadaverine and CD-1 mice showed that concentration levels of the inhibitor which produced even the slightest evidence of toxicity far exceeded the dose level necessary to inhibit microfilariae production *in vitro*.

In vivo experiments have been performed in mice with marked success. The described transglutaminase inhibitors and pseudo-substrates as well as the methods of using them are therefore quite likely to provide treatment of filariasis in other mammalian hosts. By way of example, this method should be acceptable in cats, dogs, primates and especially humans,

for treatment of filarial infections.

In vivo experiments were performed by administering the transglutaminase inhibitor, monodansyl cadaverine, intravenously to a mammalian host. However, any type of parenteral mode of administration, such as intravascular, intraarterial, or even *via* a transdermal patch, is reasonably calculated to be at least as effective a means of drug delivery. Another possible mode of administration of a transglutaminase inhibitor could be enteral. Gastrointestinal tract breakdown of transglutaminase inhibitors is not an expected consequence of *in vivo* administration and it should be readily absorbed into the circulatory system.

Acceptable pharmaceutical formulations with a compatible therapeutic regimen of a transglutaminase inhibitor, such as monodansyl cadaverine, can be developed without an undue amount of experimentation by those of skill in the pharmaceutical and medical arts with the aid of the present disclosure.

The following examples are presented to describe preferred embodiments and utilities of the present invention but should not be construed as limiting the claims thereof.

EXAMPLE I
IN VITRO INHIBITION OF THE FORMATION OF MICROFILARIAE

A novel transglutaminase was identified in adult filarial nematodes of *Brugia malayi*. Incubation of the gravid female nematodes in the presence of various possible pseudo-substrates (inhibitors) for the novel transglutaminase were studied not only in *Brugia malayi* but in *Brugia pahangi and Brugia patei* as well. The following example sets forth the preferred *in vitro* method for blocking the formation of microfilariae in adult female filarial nematodes.

A. Effect of Known Pseudo-substrates for Transglutaminases on Adult Female Nematode Viability

Various known pseudo-substrates (inhibitors) for transglutaminases were investigated for their effect on viability of adult female *Brugia malayi* nematodes. Varying concentrations of each pseudo-substrate were tested over a four-day period.

Adult female *Brugia malayi* filarial nematodes were obtained from the peritoneal cavities of 120-day old infected Mongolian jirds, (*Meriones unguiculatus*), rinsed thoroughly in RPMI 1640 medium which was supplemented with 10% fetal calf serum, 20 mM Hepes buffer, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Two female nematodes were incubated, each, in one milliliter of this medium containing the desired concentration of the transglutaminase pseudo-substrate (inhibitor) for the required number of days at 37°C in an atmosphere of 5% carbon dioxide and 95% air. Adult *Brugia malayi* nematodes were examined daily for a period of 4 days for viability. Various pseudo-substrates: monodansyl cadaverine (MDC)(5), a known high affinity pseudo-substrate of transglutaminase; dimethyl-dansyl cadaverine (DDC), an inactive analogue of MDC; methylamine; putrescine and histamine, were tested at varying concentrations. As Table II indicates, only monodansyl cadaverine, at about 300 μM concentration, and putrescine, at about 20 mM concentration, produced death in the female nematodes. Each experiment was performed in duplicate.

TABLE II

Effect of MDC, DDC, Methylamine, Putrescine and Histamine on *Brugia malayi* adult female nematode viability *in vitro*.

Conc.	MDC				DDC				
Day	1	2	3	4	Day	1	2	3	4
50 µM	++	++	++	++		++	++	++	+
100 µM	++	++	++	++		++	++	++	+
200 µM	++	++	++	++		++	++	++	+
300 µM	-	-	-	-		++	++	++	+
500 µM	-	-	-	-		N.D.			

—

Conc.	Methylamine				Putrescine				Histamine			
Day	1	2	3	4	1	2	3	4	1	2	3	4
5 mM	++	++	++	++	++	++	++	++	++	++	++	+
10 mM	++	++	++	++	++	++	++	++	++	++	++	+
20 mM	++	++	++	++	+	-	-	-	+	+	+	+

Grading: ++, Alive and very active; +, Alive but inactive (Less mobile); -, completely immobile

Inhibitors were used at the required concentration in RPMI 1640 and 10% FCS (fetal calf serum).

Results from a single experiment in duplicate.

Two adult female nematodes were incubated for each concentration.

MDC - monodansyl cadaverine
DDC - dimethyl-dansylcadaverine

Adult nematodes were recovered from the peritoneal cavity of *Brugia malayi* infected jirds. Two nematodes were incubated for each concentration in duplicate at 37°C and in an atmosphere of 5% CO_2 and 95% air.

N.D. - not determined

B. **Effect of Known Pseudo-substrates for Transglutaminases on the Release of Microfilariae from *Brugia malayi***

The effect of monodansyl cadaverine (MDC), dimethyl-dansyl cadaverine (DDC), methylamine, putrescine and histamine on microfilariae release by *Brugia malayi in vitro* was studied. Adult female *Brugia malayi* nematodes obtained and treated as in Example IA were incubated in 1 ml each of the same medium either with or without varying concentrations of one of the following transglutaminase pseudo-substrates (inhibitors) or inactive analog: monodansyl cadaverine, methylamine, putrescine, histamine or dimethyl-dansyl cadaverine, the inactive analog of MDC, with or without 10% fetal calf serum.

Two nematodes were incubated for each concentration in duplicate at 37°C in an atmosphere of 5% carbon dioxide and 95% air. After a 24 hour incubation period, the parasites were removed and 10 µl of the spent medium was examined under the light microscope to count the microfilariae. A similar procedure was followed after a 48, 72 or 96 hour incubation period. The results presented at Table III are the average number of microfilariae released by two female nematodes from four independent experiments. The standard deviation from the mean was less than 10%.

TABLE III
Effect of MDC on Mf release *in vitro* by *Brugia malayi* female nematodes

MDC	10% FCS	Mf counts in 1 ml of spent medium (Mean of 2 experiments in duplicate)			
		Days of incubation			
		Day 1	Day 2	Day 3	Day 4
0 (Control)	-	3800	4000	4120	4000
	+	5400	5800	5600	5500
50 μM	-	600	670	710	760
	+	1500	1400	1500	1600
100 μM	-	300	270	260	240
	+	1600	1400	1500	1500
200 μM	-	0	0	0	0
	+	46	50	50	48
300 μM	-	0	0	0	0
	+	0	0	0	0
		(All nematodes were inactive, immobile and slowly killed)			

Adult worms were recovered from the peritoneal cavity of *Brugia malayi* infected jirds. Two worms were incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 μl of spent medium and calculated for 1 ml.
FCS- fetal calf serum
Mf - microfilariae
MDC - monodansyl cadaverine
- without 10% fetal calf serum
+ with 10% fetal calf serum

The results for monodansyl cadaverine incubation can be seen in Table III and are plotted in Figure 1A. Incubation with 200 μm concentration of monodansyl cadaverine (MDC) or greater prevents mature microfilariae production. Figure 1A inset indicates the more than 3-fold reduction of microfilariae by only a 50 μm concentration of monodansyl cadaverine.

Figure 1B and Figure 1C illustrate the effect of medium alone versus medium containing a 200 μm concentration of

monodansyl cadaverine on developing embryos *in utero* in *Brugia malayi*. Figure 1B shows the sheathed embryos differentiating in culture medium and Figure 1C shows the lack of a sheath and lack of differentiation in the developing embryos due to the presence of monodansyl cadaverine.

The results of the incubation of adult female *Brugia malayi* nematodes with dimethyl-dansyl cadaverine (DDC), a structurally related analog of MDC, in varying concentrations can be seen in Table IV. Dimethyl-dansyl cadaverine, which does not inhibit transglutaminase activity, failed to inhibit microfilariae maturation and production *in vitro* in *Brugia malayi*.

TABLE IV

Effect of DDC on Mf release *in vitro* by *Brugia malayi* female nematodes

DDC	10% FCS	Mf counts in 1ml of spent medium (Mean of 2 experiments in duplicate)			
		Days of incubation			
		Day 1	Day 2	Day 3	Day 4
0 (Control)	-	2900	3200	3300	3300
	+	2400	3600	3400	3500
50 μM	-	2200	2300	2400	2400
	+	2600	2600	2500	2300
100 μM	-	2000	2100	2000	2100
	+	2500	2600	2600	2500
200 μM	-	2400	2700	2800	2600
	+	2600	2800	2600	2500
300 μM	-	2400	2400	2300	2100
	+	2500	2300	2200	2200
500 μM		N.D.			

Adult nematodes were recovered from the peritoneal cavity of *Brugia malayi* infected jirds. Two nematodes were incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 μl of spent medium and calculated for 1 ml.
FCS - fetal calf serum
Mf - microfilariae
DDC - dimethyl-dansyl cadaverine
N.D. - not determined

The results for the incubation of adult female *Brugia malayi* nematodes with varying concentrations of methylamine, putrescine and histamine can be seen in Table V. It is felt that the data collected after Day 1 is skewed. These particular transglutaminase pseudo-substrates, after the first 24 hours, actually become physiological substrates and are metabolized by the adult nematodes and no longer function as competitive inhibitors. Histamine showed the most promising effects of this group of pseudo-substrates, but it is not suitable as a pharmaceutical treatment.

TABLE V

Effect of Methylamine, Putrescine and Histamine on Mf release *in vitro* by *Brugia malayi* adult female nematodes worms

Conc.	10% FSC	Mf counts/ml of spent medium (Mean of 2 experiments in duplicate) Days of Incubation: Day 1	mf inhibition (%/control) Day 1	Day 2	Day 3	Day 4
0(Control)	-	2400		3000	3520	3860
	+	3600	0	3840	3800	4010
Methylamine						
5 mM	-	1050		2000	2400	3280
	+	1300	64	2880	3000	3460
10 mM	-	1300		1280	1800	2240
	+	1400	61	2000	2200	2560
20 mM	-	1400		1840	1900	2100
	+	1700	53	2200	2000	2100
Putrescine						
5 mM	-	1056		1520	1720	2080
	+	1000	72	2440	3000	3760
10 mM	-	800		1120	1400	2160
	+	860	76	1280	2000	2800
20 mM and	-	200		Adults became inactive		
	+	280	92	killed		
Histamine						
5 mM	-	950		1360	2000	2840
	+	1200	67	2400	2400	3000
10 mM	-	300		720	1000	1120
	+	1250	65	1440	1500	1920
20 mM	-	100		240	260	320
	+	350	90	560	640	800

Adult nematodes were recovered from the peritoneal cavity of *Brugia malayi* infected jirds. Two nematodes incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 µl of spent medium and calculated for 1 ml.

FCS - fetal calf serum

Mf - microfilariae

\- without 10% fetal calf serum.

\+ with 10% fetal calf serum.

Thus, the ability to inhibit microfilariae maturation and production correlated well with the pseudo-substrate's ability to inhibit transglutaminase activity. Monodansyl cadaverine, the most active transglutaminase inhibitor thus far studies, was the most successful in inhibiting microfilariae maturation and production, and methylamine, the least efficient transglutaminase inhibitor, was least successful in inhibiting production and maturation of microfilariae.

C. Effect of Pretreatment with Monodansyl Cadaverine on Microfilariae Release *in Vitro* in *Brugia malayi*

Brugia malayi were pretreated with 200 μm monodansyl cadaverine for 24 hours in the RPMI 1640 medium recited in Example IA. Pretreated *Brugia malayi* were then transferred to normal medium RPMI 1640 for 1 to 4 days without the monodansyl cadaverine.

The results of the pretreatment experiment can be seen in Table VI. This experiment indicates that so long as the nematodes are exposed to these non-toxic concentrations of monodansyl cadaverine, the release and production of microfilariae are inhibited. Once the nematodes are returned to normal medium they begin to release microfilariae, irrespective of the period of incubation or pretreatment. As the pseudo-substrate is gradually removed in normal RPMI 1640 medium, transglutaminase inhibition is removed and embryological development of microfilariae resumes.

TABLE VI

Effect of pre-incubation of *Brugia malayi* adult nematodes *in vitro* in MDC (200 uM concentration) and further release of Mf in normal medium (RPMI 1640).

Pre-incubation time	10% FCS	Mf release *in vitro*/ml Days after incubation			
		Day 1	Day 2	Day 3	Day 4
24 hr	-	300	440	1200	1600
	+	700	760	1450	1900
48 hr	-	320	640	800	1000
	+	500	600	1640	2100
72 hr	-	100	240	560	600
	+	220	430	800	840
96 hr	-	90	180	200	380
	+	120	160	280	440

Adult worms were recovered from the peritoneal cavity of *Brugia malayi* infected jirds. Two worms were incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 µl of spent medium and calculated for 1 ml.
Results from two experiments in duplicate.
FCS - fetal calf serum
Mf - microfilariae
MDC - monodansyl cadaverine

D. **Effect of Monodansyl Cadaverine on Microfilariae Release *In Vitro* by *Brugia pahangi* Adult Female Nematodes**

Adult female *Brugia pahangi* nematodes were obtained from the peritoneal cavities of 120-day-old infected jirds, rinsed thoroughly in RPMI 1640 medium which was supplemented with 10% fetal calf serum (FCS), 20 mM hepes buffer, 100 µg/ml of penicillin and 100 µg/ml of streptomycin. Two female nematodes were incubated, for each concentration of monodansyl cadaverine, either 100 or 200 µm, in this supplemented RPMI 1640 medium. The control nematodes, without monodansyl cadaverine, and the nematodes in one milliliter of the supplemented RPMI 1640 medium plus the monodansyl cadaverine were incubated for a period of 1 to 4 days at 37°C in an atmosphere of 5% carbon dioxide and 95% air. Microfilariae counts were taken after the 1 to 4 day incubation in 10 µl

aliquots of spent medium and calculated for 1 ml. Data presented in Table VII is a mean of two experiments in duplicate.

The results of this experiment can be seen in Table VII. These dates demonstrate a 200 μm concentration of monodansyl cadaverine inhibits the production of microfilariae in *Brugia pahangi* after the first day of treatment, *in vitro*.

TABLE VII
Effect of MDC on Microfilariae release *in vitro* by *Brugia pahangi* adult female nematodes

		Mf counts in 1 ml of spent medium (Mean of 2 experiments in duplicate)			
		Days of incubation			
MDC	10% FCS	Day 1	Day 2	Day 3	Day 4
0(Controls)	-	2800	3200	3100	3100
	+	4200	4400	4400	4500
100 uM	-	500	540	560	500
	+	1000	980	1000	1100
200 uM	-	0	0	0	0
	+	20	28	30	30

Adult nematodes were recovered from the peritoneal cavity of *Brugia pahangi* infected jirds. Two nematodes were incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 μl of spent medium and calculated for 1 ml.
FCS - fetal calf serum
Mf - microfilariae
MDC - monodansyl cadaverine

E. Effect of Monodansyl Cadaverine on Microfilariae Release *in vitro* by *Brugia patei* Adult Female Nematodes

Adult female *Brugia patei* nematodes were obtained and treated as recited in Section D.

The results can be seen in Table VIII. Incubation of *Brugia patei* adult female nematodes with monodansyl cadaverine at the 200 μM concentration level prevents the release and production of mature microfilariae of *Brugia patei*, *in vitro* after 24 hours.

TABLE VIII
Effect of MDC on Mf release *in vitro* by *Brugia patei* adult female nematodes

		Mf counts in 1 ml of spent medium (Mean of 2 experiments in duplicate)			
		Days of incubation			
MDC	10% FCS	Day 1	Day 2	Day 3	Day 4
0 (Controls)	-	900	920	950	930
	+	1100	1200	1220	1200
100 μM	-	600	640	620	640
	+	800	820	830	850
200 μM	-	0	0	0	0
	+	14	24	26	25

Adult nematodes were recovered from the peritoneal cavity of *Brugia patei* infected jirds. Two nematodes were incubated for each concentration in duplicate and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Mf counts were taken in 10 μl of spent medium and calculated for 1 ml.
FCS - fetal calf serum
Mf - microfilariae
MDC - monodansyl cadaverine

F. Detection of Transglutaminase in Adult Female *Brugia malayi* Nematodes by Western Blot Analysis

Adult nematodes were obtained from the peritoneal cavities of infected Mongolian jirds as described in Section A. 15 male and 15 female nematodes were immediately rinsed three times in 20 mM Tris-buffered saline (pH 7.6), sonicated for 30 seconds in 400 μl (microliters) of the same Tris buffer containing 1 mM EDTA, 150 mM sodium chloride, 1.5 mM β-mercaptoethanol and 1 mM phenylmethylsulphonyl fluoride. The sonicates were microfuged for 5 minutes and the supernatants

were recovered and referred to as the soluble fraction. The pellet was resuspended in 200 µl of 0.1% (v/v) zwitterionic detergent, 3-[(3-chloramidopropyl)dimethylamino]-1-propane sulfonate (CHAPS) in Tris-buffered saline, sonicated and referred to as the insoluble fraction.

The soluble and insoluble fractions were each mixed with SDS sample buffer containing 5% ß-mercaptoethanol and boiled. 50 µg fraction samples were electrophoresed on a 4 to 20% polylyacrylamide-gradient gel and then transferred to nitrocellulose. Immunodetection of transglutaminase on nitrocellulose paper was performed using as anti-tissue transglutaminase monoclonal antibody (CUB 7401) and alkaline-phosphatase-coupled goat antibody to mouse immunoglobulin G (Promega) as second antibody (7). Lane 1: MW markers; Lane 2: one µg of purified guinea pig liver transglutaminase; Lane 3: extracts from male nematodes; Lane 4: soluble extracts from female nematodes; Lane 5: insoluble extracts from female nematodes.

The results of the immunoblot analysis of male and female nematode proteins can be seen in Figure 2A. Monoclonal antibody (CUB 7401) against tissue-type transglutaminase revealed the presence of a single major immunoreactive band at 22 kD in the soluble extracts from female nematodes, as seen in Figure 2A, Lane 4. Immunoblots of the insoluble fraction from the female nematodes reacted weakly but consistently with anti-transglutaminase antibody. Extracts from male nematodes showed no detectable band, indicating a lack of transglutaminase enzyme, Figure 2A, Lane 3.

G. Detection of Transglutaminase in Adult Female *Brugia malayi* Nematodes by Immunoprecipitation

Male and female *Brugia malayi* nematodes, freshly obtained as in Section A, were metabolically labeled by culture for one hour in methionine/cysteine-free medium containing 200 μCi/ml of Tran-[^{35}S] label (ICN Biomedicals). The labeled parasite extracts were immunoprecipitated with CUB 7401 antibody and the immune-complexes were fractionated by SDS-gel electrophoresis, and detected by fluorography. Figure 2B, Lane 1: immunoprecipitate from male nematodes; Lane 2: immunoprecipitate from female nematodes; Lane 3: insoluble extracts from female nematodes.

Immunoprecipitates from adult nematodes pulse labeled with [^{35}S] methionine confirmed the identity and pattern of transglutaminase in the soluble and insoluble fractions of female nematodes, whereas the immunoprecipitate from the male nematodes showed no detectable band at the 22 kD position.

H. Total Protein Profiles of Adult *Brugia malayi* Nematodes

Adult *Brugia malayi* nematodes were obtained as described in Section A. Adult nematodes were treated as described in Section F to obtain the soluble and insoluble fractions. These fractions were each mixed with SDS sample buffer containing 5% β-mercaptoethanol and boiled. 50 μg of each were electrophoresed on a 4 to 20% continuous PAGE, followed by protein staining with Coomassie blue. Figure 2C illustrates the results. Lane 1: 5 μg of purified liver transglutaminase; Lane 2: adult male nematodes; Lane 3: adult female nematodes soluble fraction; Lane 4: adult female nematodes insoluble fraction; Lane 5: molecular weight markers.

Comparison of the Coomassie blue profile of male and female adult nematodes (Figure 2C) revealed some differences

in the protein bands; therefore differences in total protein profiles of the two sexes exists.

I. Indirect Immunofluorescence Labeling of *Brugia malayi* Female Nematodes

Nematode specimens freshly obtained as detailed in Section A were immediately fixed on slides in pre-chilled ethanol:acetic acid (3:1) for five minutes, permeablized with 0.1% CHAPS in phosphate buffered saline (PBS), stained with monoclonal antibody to tissue transglutaminase (CUB 7401), followed by a fluorescein isothiocyanate (FITC)-labeled goat antibody to mouse immunoglobulin G (Sigma). A drop of p-phenylene-diamine in a mixture of PBS and glycerol (1:9) was added and cover slips were applied. The slides were examined under a Leitz phase-contrast, Figure 3A, and a fluorescence microscope, Figure 3B, using a 40X objective.

Indirect immunofluorescence staining of female nematodes, Figure 3B, with CUB 7401 antibody followed by a FITC-conjugated anti-mouse IgG, elicited a distinct immunofluorescence pattern. In control experiments, normal mouse serum, supernatant from a control hybridoma clone CUB 11 or second antibody alone failed to produce immunofluorescence (data not shown).

J. Detection of Substrate Proteins Used by Exogenous and Endogenous Transglutaminases

Freshly obtained male and female *Brugia malayi* adult nematodes as described in Section A were sonicated in buffer solution as described in Section F. Figure 4A illustrates detection of substrate proteins for various transglutaminases. 250 μg aliquots from male (Lane 2 and Lane 4) and female (Lane 3 and Lane 5) extracts were incubated with 5 μg of liver (exogenous) transglutaminase (Lane 2 and Lane 3) at 37°C in a final volume of 200 μl reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 300 mM sodium chloride, 15 mM β-mercaptoethanol, 200 μM monodansyl cadaverine and 5 mM of

either calcium chloride (Lane 2 and Lane 3) or EGTA (Lane 4 and Lane 5). After 30 minutes incubation, the reaction was stopped by the addition of SDS-sample buffer. The samples were boiled, and 50 (Lane 2 and Lane 3) or 100 (Lane 4 and Lane 5) μg of the extract were separated on a 10% SDS-polyacrylamide gel.

Transglutaminase-catalyzed conjugation of proteins to monodansyl cadaverine was visualized under UV light. Proteins which served as substrates for endogenous transglutaminase (Lane 6) were detected by incubating the freshly isolated female nematodes for 24 hours at 37°C in RPMI 1640 medium containing 10% FCS with or without 200 μM monodansyl cadaverine. At the end of the incubation period, the nematodes were removed and thoroughly washed in Tris buffer (20 mM, pH 2.5), sonicated for 30 seconds and microfuged. The supernatants were then subjected to SDS-PAGE and the proteins which were covalently conjugated to monodansyl cadaverine were visualized under UV light.[8] Extracts from untreated female nematodes served as the control (Lane 7). Lane 1 contains the molecular weight markers. Figure 4B is the Coomassie blue stained gel of the protein sample shown in Figure 4A.

Results from this study are shown in Figure 4A and 4B. Extracts from male and female filarial nematodes contained several proteins which served as substrates for purified liver tissue transglutaminase. Nevertheless, in female filarial nematodes the number and amount of substrate proteins was much higher than those present in male filarial nematodes (Figure 4, Lane 3 versus Lane 2). Conjugation of monodansyl cadaverine to nematode proteins was completely blocked in the presence of EGTA (Figure 4, Lane 4 and Lane 5), suggesting the requirement for calcium ions (Ca^{+2}) for the enzymatic activity. In order to identify the parasitic filarial nematode proteins used as substrates by endogenous transglutaminase, the *in situ* conjugation of monodansyl cadaverine in live female nematodes

was studied. Fractionation of extracts from monodansyl cadaverine treated female nematodes on SDS-PAGE and visualization under UV light, revealed the presence of a major monodansyl cadaverine conjugated fluorescent band at 65 kD (Figure 4, Lane 6). In addition, several other minor bands were detected ranging from 15 kD to 150 kD molecular weight. The bands at 21, 42, 45, 55 and 130 kD were relatively prominent. Control extracts from untreated female nematodes failed to show any fluorescent band under UV light (Figure 4A, Lane 7).

EXAMPLE II

IN VIVO TRANSGLUTAMINASE INHIBITION IN *BRUGIA MALAYI*

Preliminary studies utilizing a transglutaminase inhibitor, monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] to treat *Brugia malayi* nematodes within a mammalian host indicated that the filarial infection can be controlled, if not eliminated, in the host.

A monodansyl cadaverine toxicity study was performed which showed that levels of the transglutaminase inhibitor far exceed the desired dosage necessary to treat the filarial infection in humans or other animals, produced little if any host toxicity, and were filaricidal.

For treatment, the transglutaminase inhibitor, preferably monodansyl cadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide], but not limited to only this pseudo-substrate, may be formulated into pharmaceutical compositions and administered using a compatible therapeutic regimen. With the aid of the present disclosure, those of skill in the art would be able to derive suitable dosages and schedules of administration for any number of effective compositions containing a transglutaminase inhibitor. A preferred dosage,

at least for MDC, might be that which is sufficient to achieve an effective blood concentration of greater than about a 300 μM level. The dosage in an average person might be in the range of no more than 6 mg/Kg body weight. Determination of effective amounts of any particular transglutaminase inhibitor, however, should be determinable by one of ordinary skill in the art given the present application.

In addition to the transglutaminase inhibitor, the pharmaceutical compositions may contain any number of acceptable pharmaceutical excipients and auxiliaries that facilitate process of the active compounds into the preparations that can be used pharmaceutically. As previously indicated, preparations may be designated for parenteral, transdermal or enteral administration to mammalian hosts, e.g. humans. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water soluble or water dispersible form. Sometimes, suspensions of active compounds may be administered in suitable lipophilic carriers, one example being liposmones. The pharmaceutical formulations may contain substances which increase viscosity, for example, sodium carboxymethyl cellulose, sorbitol and/or dextran. Optionally, the formulation may also contain stabilizers. With each formulation, suitable excipients such as saline, lipids or physiologic buffers, known to those of skill in the art, may be used.

A. *In Vivo* Effect of Monodansyl Cadaverine on Survival of *Brugia malayi* Adult Nematodes in a Mammalian Host

The effect of intraperitoneal doses of monodansyl cadaverine on *Brugia malayi* adult female nematodes which were transplanted intraperitoneally into nude mice was studied. Six nude mice were taken as the control group and each transplanted intraperitoneally with five adult female *Brugia malayi* nematodes. Four weeks after the transplantation the animals were sacrificed and the nematodes recovered. For the

monodansyl cadaverine test group, eight nude mice were each transplanted intraperitoneally with five *Brugia malayi* adult female nematodes. A week after transplantation, these animals were treated intraperitoneally with monodansyl cadaverine (0.2 ml of a 10 mM solution) three times per week for one week. Two weeks after the last monodansyl cadaverine treatment the animals were sacrificed and the nematodes were recovered and counted.

A significant difference in the number of viable nematodes existed between the control group and the group treated with monodansyl cadaverine. Some nematode loss may be accorded to natural rejection, about 1.5 fold difference in the original number of nematodes transplanted per animal versus the number of nematodes per animal at the completion of the experiment. This does not account for the 13-fold difference in the number of nematodes in the control animals versus the number of nematodes per animal treated. Monodansyl cadaverine is demonstrated by the data to be very effective in reducing the viability of the *Brugia malayi* nematodes. These results can be seen in Table IX. The concentration of monodansyl cadaverine administered to each mouse was about 5 times greater than the concentration necessary, *in vitro*, to prevent microfilariae development *in utero* in the adult *Brugia malayi* female nematodes.

TABLE IX

In vivo effect of MDC on survival of *Brugia malayi* adult Nematodes

Treatment with MDC (I/P)	Animals	No. of Total Nematodes	X Nematodes/ Animal
None (Control)	6	20 (4/6)*	3.3
days 1, 3, 5	8	2 (2/8)*	0.25

*Number of animals with adult nematodes per number of animals tested.

5 adult nematodes each were transplanted into nude mice intraperitoneally (I/P). A week after the transplantation, animals were injected intraperitoneally with MDC (0.2 ml of 10 mM solution) three times in one week on alternate days. Two weeks after the last treatment, animals were sacrificed and nematodes were recovered and counted.
MDC = monodansyl cadaverine

B. *In Vivo* Toxicity of Monodansyl Cadaverine in CD-1 Mice

Four groups of four male CD-1 mice were studied for toxic reactions due to administration of varying amounts of monodansyl cadaverine (MDC) either intravenously or intraperitoneally (Table X).

TABLE X

In vivo Toxicity Study of MDC in CD-1 Mice

Group (4 mice each)	Amount of 10mM MDC	IV or IP	mg MDC or mg mouse	MDC/Kg body weight	Toxic Reaction
1	0.2 ml	IV	0.670	14.80	NONE
2	0.3 ml	IV	1.000	22.30	NONE
3	0.5	IV	1.675	36.22	ACTIVITY DECLINE FOR FEW SECONDS
4	0.7	IP	2.345	52.10	NONE

MDC - monodansyl cadaverine
IV - intravenously
IP - intraperitoneally

Each mouse was four months old and weighed 40 to 50 grams. Measured aliquots were taken from a 10 mM stock solution of monodansyl cadaverine in saline, pH 2.5, and injected into the mice. Group 1 mice were given a 0.2 ml bolus intravenously of the 10 mM stock solution. This dose is equivalent to 0.670 mg MDC per mouse or about 14.8 mg/Kg of body weight. No toxicity was observed at this dose in any of the animals. Group 2 mice were given a 0.3 ml bolus intravenously of the 10 mM MDC stock solution. This dose is equivalent to 1.0 mg MDC per mouse or about 22.3 mg/Kg of body weight. No toxicity was observed at this dose in any of the animals. Group 3 mice were given a 0.5 ml bolus of the 10 mM stock solution intravenously. This dose is equivalent to 1.675 mg MDC per mouse or about 37.22 mg per Kg of body weight. Immediately after this injection the animals showed a definite decline in activity but recovered normal activity rate within a few seconds and no deaths were observed. Group 4 mice were injected with a 0.7 ml bolus of the 10 mM MDC stock solution intraperitoneally. This dose was equivalent to 2.345 mg MDC or mouse or 52.10 mg MDC/Kg of body weight. No toxic effect was demonstrated at this dose.

The results of the *in vivo* toxicity study indicated that the concentration of monodansyl cadaverine necessary to inhibit microfilariae production in adult female filarial nematodes is about five times less than the minimum dose used in the toxicity study which had no adverse effects on the mammalian host. The dose range of monodansyl cadaverine necessary to be filaricidal is also less than the minimum dose range for the toxicity study.

EXAMPLE III

IDENTIFICATION OF TGASE ENZYME SUBSTRATES IN NEMATODES

The identification of biochemical pathways or enzymes that are parasite-specific and vital for growth and survival

of nematodes will help pinpoint the possible sites of attack for model nematode inhibitors. In the present example, the presence of a transglutaminase enzyme and its substrate proteins in male and female worms is identified. Substrate proteins are identified in the present example using purified guinea-pig liver tissue TGase.

In this example, it is shown that both the male and female filarial worms contain several proteins that could serve as suitable substrates for an exogenous or endogenous transglutaminase enzyme. Higher levels of these suitable substrates, both quantitatively and qualitatively, are demonstrated in female worms as compared to male worms. Transglutaminase-catalyzed reactions appear to play an important role during embryogenesis in filarial parasites. This is supported by the data presented herein of the high concentrations of transglutaminase enzyme observed in developing embryos inside female worms (see Example IV). The amount of Ca^{+2}-dependent covalent incorporation of [^{3}H] putrescine into parasite proteins was determined as described *infra*.

Parasites: Adult male and female worms of *B. malayi* were recovered from the peritoneal cavities of jirds 120-150 days post-infection with third-stage infective larvae (L_3). Worms were washed twice with sterile saline and cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (referred to as "medium"). *In utero* developing embryos were recovered by dissecting the female worms with sharp surgical blades in the presence of medium. The damaged parasites were incubated at 37°C for 45-60 min to allow maximum expulsion of the embryos. Uterine contents thus obtained were centrifuged and the pellet resuspended in a minimal volume of the medium.

Isolation of sheaths: Developing embryos isolated from adult

female worms were pelleted by centrifugation, and the pellet was resuspended in 0.5 ml of 10% (w/v) SDS in Tris-buffer (20 mM, pH 7.4), containing 150 mM NaCl and 250 mM ß-mercaptoethanol (2-ME). The pellet was treated two more times with SDS as in the first step and the final pellet, which contained pure sheaths, was resuspended in 0.1 ml of the buffer for examination under the microscope.

Detection of pTGase in parasite extracts: Male and female worms were separated, rinsed three times in 20 mM Tris-buffered saline (pH 7.6), and lysed immediately in the cold by sonication in the same buffer containing 150 mM NaCl, 1 mM EDTA, 1.5 mM 2-ME, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Worm extracts were centrifuged (10,000 *g* for 10 min); the supernatants were designated the "soluble fraction". The pellet was resuspended in the same buffer containing 0.2% (v/v) zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and sonicated; it was designated the "insoluble fraction." The soluble and insoluble fractions were mixed with SDS-sample buffer, boiled, and fractionated by electrophoresis on a 4-20% polyacrylamide-gradient gel and then transferred onto nitrocellulose paper. The nitrocellulose paper was neutralized with 1% bovine serum albumin (BSA), and TGase was detected by a method described earlier[7], using mouse monoclonal antibody (CUB 7401) produced against guinea-pig liver tissue TGase[15] and alkaline phosphatase-conjugated goat antibody to mouse immunoglobulin G (Promega Corp., Madison, WI) as second antibody.

Metabolic Labeling and Immunoprecipitation of pTGase: Male and female worms were separated and cultured in methionine/cysteine-free medium (GIBCO Labs., Grand Island, NY) containing 200 μCi ml^{-1} of Tran-[^{35}S] label (sp. act. 1, 197 Ci/mmol; ICN Labs., Costa Mesa, CA). After a 45 min pulse at 37°C, the parasites were removed and washed twice with

phosphate-buffered saline (PBS) and resuspended in Tris-HCl (20 mM; pH 7.5, containing protease inhibitors). The parasites were lysed by sonication and equivalent aliquots (2 x 10^6 cpm) of labeled parasite extracts were immunoprecipitated by incubation for 4 h at 4°C with CUB 7401 monoclonal antibody against tissue TGase (2.5 µg). Immune complexes were sedimented by adding 300 µl RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and 20 µl of a 10% suspension of fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem, San Diego, CA)[16]. The precipitated complexes were washed with RIPA and the proteins were solubilized in an SDS-containing electrophoresis buffer. The samples were fractionated by SDS-gel electrophoresis on a 4-20% continuous gradient slab gel, and the radiolabeled proteins were located by fluorography under conditions described in detail previously.[17]

Indirect immunofluorescence staining: Freshly isolated female worms were cut into small pieces with a surgical blade, and the liberated uterine contents and adult worm-debris were smeared on clean glass slides. After air dying, the smears were fixed with methanol at -20°C for 15 min. After extensive washing with PBS, they were incubated with either undiluted CUB 7401 monoclonal antibody supernatant or with supernatant from a control hybridoma (CUB 11).[7] The second antibody in each case was a 1:1,000 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (γ-chain specific; Sigma, St. Louis, MO). The stained slides were examined under a Nikon fluorescence microscope using transmitted dark field illumination and a 40 x objective. Pictures were taken on Kodak X-pan film using an exposure time of 2 min.

Fluorescence staining of pTGase substrate proteins: Female worms were incubated in RPMI-medium with or without 200 µM monodansylcadaverine (MDC, Sigma) for 24 h. At the end of incubation, parasites were rinsed in PBS and the uterine

contents, obtained by damaging the worms, were smeared on glass slides, and air dried. Unbound MDC from the parasites was eluted by extensive washing with chilled 20% acetic acid and 30% methanol in water for 3 h. The pH was brought to neutral (about pH 7) by washing the slides with several changes of Tris-buffer (20 mM, pH 7.4) containing 150 mM NaCl. The smears were mounted in 90% glycerol (v/v) in PBS and observed under fluorescence microscope, using a Blue excitation radiation filter (B-2A, Nikon).

Determination of substrate proteins in adult worms: Parasite proteins used as substrates by exogenous TGase were determined by studying the Ca^{+2}-dependent covalent incorporation of [^{3}H] putrescine.[18,19] The reaction was carried out in 0.1 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 10 mM dithiothreitol, 0.5 μCi 2,3-[^{3}H]putrescine (29.7 Ci/mmol; New England Nuclear, Wilmington, DE), 10 μl of enzyme (5 μg purified TGase from guinea-pig liver), and 50 μl worm extracts (40-75 μg protein). Reaction mixtures were incubated for 15 min at 37°C, and the reaction was stopped by spotting aliquots of 25 μl reactants in triplicate on Whatman 3 mm filter paper. Filters were immediately fixed and washed in trichloroacetic acid and ethanol, and protein bound radioactivity was determined by liquid scintillation spectrometry. Background values were obtained by substitution of 5 mM EGTA for $CaCl_2$ in the reaction mixture. The specificity of TGase to catalyze incorporation of [^{3}H] putrescine into parasite proteins was tested by studying the ability of MDC (0.5 mM) to compete with the radioactive substrate, putrescine.

Proteins in adult worms that could be used as substrate by exogenous TGase were also identified by studying the Ca^{+2}-dependent incorporation of a fluorescent pseudosubstrate, MDC. Male and female worm extracts were incubated in the presence of purified guinea-pig liver TGase (10 μg) at 37°C in a final volume of 200 μl containing 50 mM Tris-HCl (pH 7.5), 300 mM

NaCl, 15 mM 2-ME, 400 μM MDC, and either 5 mM $CaCl_2$ or EGTA. After 30 min incubation, the reaction was stopped by addition of SDS-sample buffer. The samples were boiled, and the proteins were separated on a 10% SDS-polyacrylamide gel.[11] TGase-catalyzed conjugation of proteins to MDC was visualized by exposing the gel to illumination at 366 μM.[20]

Proteins that served as substrates for endogenous TGase (pTGase) were detected by incubating the live female worms in the presence of 200 μM MDC at 37°C for 24 h. At the end of incubation, worms were washed and lysed by sonication. The parasite extracts were fractionated by SDS/polyacrylamide gel electrophoresis, fixed in 20% (v/v) methanol:10% (v/v) acetic acid, and analyzed for fluorescent proteins under UV light (366 nm). Untreated female worms served as control.

Scanning electron microscopy: Purified sheaths were obtained from the gravid female worms as described earlier. They were immediately fixed in 2% osmium tetroxide and dehydrated in a graded series of ethanol. The fixed sheath preparations were transferred to Peldri II for critical point drying, mounted onto stubs for sputter-coating with 200°A gold-palladium (Technics, VA), and examined in a Hitachi S520 scanning electron microscope.

A. TGase Enzyme Substrate in Adult Filariae

In the present study, it was demonstrated that both male and female worms contained several proteins that could serve as suitable substrates for TGase. The TGase-catalyzed reactions were also demonstrated to be significant in *in utero* growth and differentiation of embryos to mature Mf.

Since natural polyamines can undergo a variety of metabolic reactions synthetic MDC was employed in order to determine the presence of substrate proteins in adult worms. MDC is a good general amine donor for TGases of all types.[13]

Results shown in Table XI demonstrate that adult worms contain large amounts of proteins that could serve as suitable substrates for TGase.

TABLE XI

Incorporation of [2, 3-^{3}H(N)]-putrescine into *B. malayi* adult worm proteins by guinea-pig liver TGase.

Incubation[a] protein)[b]	[^{3}H]putrescine incorporation (cpm/25μg	
	Female worms	Male worms
Casein		
Expt. 1		
Ca^{+2} (5mM)	23,047	ND2,939
EGTA (5 mM)	358	ND279
Ca^{+2} (5 mM) + MDC (0.5 mM)	2,255	ND1,272
Expt. 2		
Ca^{+2} (5 mM)	37,959	14,2333,886
EGTA (5 mM)	421	224348
Ca^{+2} (5 mM) + MDC (1 mM)	2,141	9241,170
Ca^{+2} (5 mM) + MDC (1 mM)	38,780	13,612ND

[a] In a 100 μl final volume the reaction mixture contained 20 mM Tris-buffer (pH 7.4), 150 mM NaCl, 15 mM 2-ME, Ca^{+2}/EGTA and MDC at appropriate concentrations, 0.5 μCi [^{3}H]putrescine (29.7 Ci/mmol sp act), and 5 μg TGase (purified from guinea-pig liver) and the parasite extract or dimethyl casein. After 30 min, the reaction was stopped by spotting 25 -μl reactants in triplicate on Whatman filter and immersing them immediately in 10% cold TCA, followed by two washes in 5% TCA as described in Materials and Methods.

[b] An average of triplicate values. Standard deviation from the mean was less than 10%

ND - Not determined

The rate of [^{3}H] putrescine incorporation into parasite proteins was found to be linear for at least the first 15 min

autoradiography confirmed the identity and pattern of pTGase in soluble and insoluble extracts of female worms (Fig. 2B, lanes 2 and 3). Immunoprecipitates from male worms showed no detectable band (Fig. 2B, lane 1).

From the band intensity on immunoblots, it is postulated that TGase in female worms constitutes 0.2-0.3% of the total parasite protein. Affinity-labeling with MDC demonstrates that developing embryos inside the female worms, especially during early stages of development, contain large amounts of pTGase.

B. TGase Enzyme Substrate in Filarial Embryo *in utero* Differentiation and Embryogenesis

The present example demonstrates that the inhibition of enzyme activity by TGase substrate inhibitors will effectively block *in utero* growth and differentiation of embryos to mature Mf. It is postulated that such is the result of the inhibition of sheath synthesis. It is postulated that TGase in filarial parasites is responsible for cross-linking of host-parasite proteins, and that these pTGase-catalyzed posttranslational modification of proteins is important in the assembly of egg shell or sheath.

When disrupted by boiling in SDS and 2-ME, developing embryos isolated from the uteri of *B. malayi* female worms contained mainly the sheaths in the form of smooth spherical structures (Fig. 5). When viewed under phase-contrast microscope, the sheaths looked very similar to cornified envelopes, formed by keratinocyte TGase in epidermis[22], and to apoptotic bodies, formed by TGase during programmed cell death.[23] In order to determine whether egg shell or sheath in embryos and Mf (microfilaria) of the filarial parasites was a product of TGase-catalyzed reaction, filarial worms were examined for the presence of this enzyme.

Indirect-immunofluorescent staining of uterine contents from the female worms with anti-TGase antibody and fluorescein-conjugated second antibody revealed that embryos in early stages of development are highly rich in TGase enzyme (Fig. 6). Fully developed or developing embryos in their advanced stages of differentiation towards mature Mf contained less of the enzyme peptide, as suggested by the observed poor immunofluorescence intensity.

C. Fluorescence Microscopy of Embryonic Filarial Proteins as TGase Substrates

The above-described pattern of relative distribution for pTGase during embryonic stages of filarial parasites was confirmed by *in situ* labeling of the female worms with a fluorescent TGase-substrate, MDC. Female worms were incubated at 37°C in the presence of MDC in order to allow the endogenous enzyme to conjugate MDC to the parasite substrate proteins. At the end of incubation, unbound MDC was eluted, and worms were processed for fluorescence microscopy.

As revealed by fluorescence intensity, embryos during early developmental stages incorporated large amounts of MDC (Fig. 7), suggesting the presence of TGase in its active form. Female worms incubated in the absence of MDC showed no or very little autofluorescence.

EXAMPLE IV

TRANSGLUTAMINASE-INHIBITION IN GROWTH AND DIFFERENTIATION OF *B. MALAYI*, *B. PAHANGI* AND *B. PATEI*

The present example is presented to further illustrate the important role of TGase-catalyzed reactions in both adult and *in utero* growth and differentiation in a representative number of different nematode species. In these studies, a variety of enzyme-specific, pseudo-substrates of transglutaminase, including by way of example, monodansylcadaverine, methylamine, histamine, and putrescine,

were used to examine the involvement of transglutaminase-catalyzed reactions in microfilariae production and release.

The described transglutaminase inhibiting agents were found to significantly inhibit microfilarial production and release by gravid female worms in a dose-dependent manner. However, the structurally related inactive analogue of monodansylcadaverine (i.e., dimethyldansylcadaverine) at an equimolar concentration, did not affect the microfilariae release by female worms of any of the *Brugia* species examined.

Reagents: Reagents employed in the present study were the same as those described in Example I B.

Parasites. Adult worms of *B. malayi*, *B. pahangi* and *B. patei* were obtained from the peritoneal cavities of Mangolian jirds, *Meriones unguiculatus*, 120-150 days after infection with third stage infective larvae of their respective species (L_3). The parasites were washed twice in sterile saline prior to their culture in medium. Developing embryos from the female worms were obtained by dissecting the parasites as described in Example I B.

Effect of enzyme inhibition on Mf release. Same as described in Example I B. Results shown at Table XII for each time point and each inhibitor concentration are the average number of mf released by two female worms in at least two independent experiments performed in duplicate.

TABLE XII

Effect of TGase inhibition on mf production and release by filarial female worms.

Species	Inhibitor	Concentration (μM)	Mf release[a] (24h)	Mf inhibition (% of control)
B. malayi				
	None	-	3,8000	
	MDC[b]	50	60084	
		100	30092	
		200	0100	
	DDC[c]	200	3,6005	
		300	3,7102	
B. pahangi				
	None	-	4,2000	
	MDC	100	1,00076	
		200	2099	
B. patei				
	None	-	1,9000	
	MDC	100	60069	
		200	1499	

[a] Total number of mf released by two female worms in 24 h period. Values are an average of two independent experiments in duplicate.
[b] MDC: Monodansylcadaverine, a TGase inhibitor [30].
[c] DDC: Dimethyldansylcadaverine, a structurally related but inactive analogue of MDC.

Scanning electron microscopy. Same as described in Example I B.

Post-translational changes in parasite proteins. Female worms of *B. malayi* were separated and cultured for 15 min in methionine/cysteine-free medium (GIBCO Labs., Grand Island, NY). After 15 min, the cultures were pulse-labeled with 100 μCi ml^{-1} of Tran-[^{35}S] label (sp. act. 1,197 Ci/mmol) for 45 min. The labeled parasites were washed thoroughly in RPMI 1640 medium and further incubated in the presence of serum-containing medium. At various time intervals, three worms each were removed and immediately sonicated in Tris-HCl buffer (20 mM, pH 7.5; containing 150 mM NaCl, 1 mM EDTA, 1.5 mM β-mercaptoethanol and 1 mM each of leupeptin and phenylmethylsulfonyl fluoride). The sonicates were solubilized by boiling with sodium mongolian sulfate (SDS)-sample buffer and the samples (corresponding to approximately 5.4 x 10^4 cpm) were applied to 4% polyacrylamide-stacking gels and electrophoresed in 10% polyacrylamide-resolving gels according to the method of Laemmli[11]. Gel slabs were then dried and radioactive bands were detected by fluorography, followed by autoradiography as described earlier[12].

Autoradiographic localization of ^{35}S-labeled proteins in developing embryos. Female worms were pulse labeled with Tran-[^{35}S] label as described above. The labeled parasites were then incubated either in the presence of medium alone or medium containing MDC (200 μM) for 24 h. Uterine contents from treated and untreated female worms were obtained and smeared on glass slides, fixed in ethanol: acetic acid (30:10, v/v) at -20°C and coated with bulk emulsion (NBT-2, Eastman Kodak, Rochester, NY) diluted 1:1 with water. The glass slides bearing embryos were processed for photography according to the manufacturer's instructions. Also, radioactivity incorporated in sheaths purified form developing embryos of MDC treated and untreated female worms was determined.

RESULTS

Significant inhibition (75%) of production and release of Mf was detected with 50 μM MDC, and a 200 μM concentration of MDC completely inhibited the Mf release by female worms of all *Brugia* species tested. Concentrations of MDC (≤ 200 μM) that inhibited

Mf production had no gross toxic effect on adult worms or fully developed Mf of each species. For example, incubation of adult worms of each species in the presence of MDC at a 200 μM concentration did not affect their motility even after 6 days of culture. MDC at 100 μM concentration caused about 70% inhibition, whereas 200 μM MDC resulted in complete inhibition of Mf production and release by female worms of all species. At either concentration, MDC had no apparent toxic effect on adult worms.

At concentrations ≥ 300 μM, the adult worms of each *Brugia* species became sluggish with time, and after 24 h of culture they were immobile and eventually died. The inhibitory effect of MDC on mf production was evident even after 30 min of incubation (data not shown).

To determine if the primary amine group of MDC was essential for the observed inhibitory effect on mf release, a dimethylated analogue, $^{\alpha}$N-dimethyldansylcadaverine (DDC), which is structurally related to MDC but lacks the primary amine group, was tested (See Fig. 12 for structure of MDC and DDC). This amine group is essential for TGase activity, and DDC thus cannot serve as a substrate inhibitor for TGase. Adult female worms of each *Brugia* species were incubated in the presence of MDC or its dimethylated analogue for 24 h. The α-N-dimethylated analogue of MDC, DDC, had no effect (Table XII) on mf production and release by female worms of each *Brugia* species even at the highest concentration used (200 μM). Unlike MDC, DDC, even at higher doses (>300 μM), had no

significant effect on the viability of adult worms, and they stayed active and alive even after 72 h of culture in the presence of DDC.

Phase-contract microscopic examination of uterine contents from MDC-treated female worms of each *Brugia* species revealed that the *in utero* development of mf in these worms was severely impaired. The uteri from untreated control worms (incubated for 72 h in medium alone) contained numerous embryos at various developmental stages with well-defined sheaths surrounding them. In contrast, MDC-treated (200 μM, 72 h) female worms contained very few developing embryos, and even these were mostly undifferentiated with very poor and undefined sheaths around them. When viewed by scanning electron microscopy, control embryos appeared as spherical or globular entities with relatively smooth surface (Fig. 8A). Embryos from MDC-treated appeared as irregular, bleby structure, probably due to ill defined or missing sheaths (Fig. 8B).

To ascertain whether MDC-induced inhibition of mf release by female worms was due to the inhibition of TGase-catalyzed reactions, the effect of several other enzyme inhibitors with significant differences in their structures (methylamine, histamine, putrescine) were tested on a variety of *Brugia* species (see Fig. 12). The amount of mf inhibition resulting from each type of treatment was then determined. Results are presented at Table XIII.

TABLE XIII
Effect of various TGase inhibitors on Mf production and release by *B. malayi* female worms.

Inhibitor	Concentration (mM)	Mf release[a] (24 h)	Mf inhibition (% of control)
None	-	3,600	0
Methylamine	5	1,050	71
Histamine	5	1,200	67
Putrescine	5	1,000	73

[a] Values represent an average number of Mf released by two female worms in two independent experiments in duplicates.

All of the 3 transglutaminase inhibitors, methylamine, histamine and putrescine, were demonstrated to inhibit Mf production by at least 65% (% of control) at a 5mM concentration.

METHODS

Adult female worms of the various *Brugia* were preincubated with MDC (200 μM) for the indicated periods of time. At the end of each incubation period, the parasites were removed, washed extensively, and resuspended in fresh medium without MDC. On various days after the incubation in MDC-free medium, spent media were drawn and examined under the light microscope and the number of Mf released determined.

RESULTS

Worms of all *Brugia* species preincubated with MDC showed a significant inhibition in mf production as compared to untreated controls. However, removal of MDC from the culture medium resulted in recovery of the parasites, as determined by mf release in the spent medium. The worms preincubated for 24 h with MDC recovered much faster than those that were preincubated for 96 h. Nevertheless, once treated with MDC,

female worms could never produce mf at levels similar to untreated controls.

Post-Translational Protein Modification

To further define a role for TGase in growth and differentiation of embryos to mature mf, the pTGase-catalyzed post-translational modification of the parasite proteins was studied. Adult female worms of *B. malayi* were pulse labeled with [^{35}S]methionine/cysteine. The labeled parasites were incubated in medium at 37°C and chased for studying the modification of labeled proteins by polyacrylamide-gel electrophoresis and autoradiography.

Results shown in Fig. 10 demonstrate that the female worms contained several proteins that could undergo covalent post-translational modification. For example, the protein bands seen at approximately 25 kDa, 36 kDa, 122 kDa, 160 kDa, and 260 kDa positions after the parasites were labeled (0 h) gradually disappeared with time in culture. Conversely, a time-dependent increase in the intensity of protein bands located at approximately 23 kDa and 87 kDa positions was observed.

In the next series of experiments, the fate of the post-translationally modified proteins in intact embryos was determined. Adult female worms of *B. malayi* were metabolically labeled with (^{35}S) methionine for 45 min. The labeled parasites were subsequently incubated in the presence of medium alone or medium containing 200 μM MDC. Twenty-four hours after incubation, the uterine contents form MDC treated and untreated worms were collected and processed for autoradiography as described in Example 3.

Incorporation of labeled parasite proteins into egg shell or sheath of developing embryos and Mf was confirmed by isolating the sheaths. The uterine contents from labeled

parasites that had been incubated in the presence or absence of MDC (200 μM for 18 h) were used to isolate the sheaths. The radioactivity in sheaths was determined by scintillation counting. Sheaths isolated from the embryos of untreated female worms contained at least 4-fold higher radioactivity (7,173 cpm/10^4 embryos) than those obtained from MDC-treated female worms (1,760 cpm/10^4 embryos).

Results shown in Fig. 11 demonstrate that labeled proteins in control parasites redistributed and incorporated themselves into the sheath of the Mf during their *in utero* differentiation from the embryos. Such incorporation of radioactivity into the sheath seems to be mediated by pTGase by virtue of its ability to cross-link the labeled parasite proteins. This was supported by the observation that the parasites incubated under identical conditions, but in the presence of the enzyme inhibitor MDC, showed no such discrete redistribution and incorporation of labeled proteins into the sheath (Fig. 11A). The radioactivity was evenly distributed throughout the Mf, possibly due to inactivation of pTGase by MDC and thus rendering it unable to cross-link the labeled parasite proteins.

These data support the concept that several endogenous proteins in filarial parasites undergo TGase-catalyzed post-translational modifications during embryogenesis that result in the formation of the sheath. Also, the examples disclosed herein demonstrate that TGase-catalyzed reactions play an important role during embryogenesis in a representative number of different filarial parasites.

The various primary amines, which serve as competitive substrate inhibitors or pseudosubstrates for TGase, significantly inhibited Mf production and release by female worms of all species tested. MDC, the most potent amine pseudosubstrate for TGase[13], was an effective inhibitor of Mf

production at far lower concentrations than histamine, putrescine or methylamine. MDC, being a competitive pseudosubstrate, was required to be continuously present to exert its inhibitory effects. The parasites could be rescued, albeit with varying effects, from the inhibitory effects after MDC was removed from the cultures (Fig. 9). These findings demonstrate that the observed effects of primary amines on the parasites were embryostatic and due to inhibition of TGase-catalyzed events.

The hypothesis that pTGase-mediated covalent cross-linking of parasite proteins is an essential event for the assembly of the sheath during *in utero* differentiation of embryos to mature Mf is supported by our observations that: a) inhibition of the enzyme activity by several structurally unrelated primary amines was associated with arrest of embryo growth and differentiation to Mf (Table XIII); a structurally related inactive analogue of MDC, the most potential amine substrate inhibitor of TGase, failed to exert such an effect (Table XII); b) endogenous proteins in female worms underwent TGase-mediated covalent post-translational modifications (Fig. 10); and, c) the labeled parasite proteins were actively incorporated into the sheath in the presence of active enzyme (Fig. 11). Filarial egg shell and sheath structures are hypothesized to contain TGase-catalyzed isopeptide bonds. The date herein presented demonstrates the presence of a significant amount (640 pmol/mg protein) of ϵ (γ-glutamyl)lysine crosslinks in these structures, a product of physiologically active TGase, in *B. malayi* adult worm extracts.

Based on the data presented herein, TGase-catalyzed reactions are postulated to participate during other developmental stages of filarial and other parasites.

It was also observed that levels of collagen synthesis,

as determined by [^{3}H]proline incorporation, in adult filarial worms[34] corresponded to the pTGase levels in these parasites. Thus, adult male worms that lack pTGase are postulated to also synthesize negligible amounts of collagen. In contrast, female worms with high levels of pTGase synthesize and produce extremely high amounts of collagen. pTGase-catalyzed cross-linking of collagen and other proteins may be essential for the formation of new cuticular surface area, which is needed during the L_4 - adult moult. Therefore, the present invention is intended to encompass methods of killing nematodes through the inhibition of cuticle formation.

EXAMPLE V
DETERMINATION OF ε(γ-GLUTAMYL)LYSINE (GLU-LYS) CROSSLINK

The analysis described in the present example was performed using the method described by Tarcsa *et al*, which reference is specifically incorporated herein by reference for this purpose.[10]

The filarial sample was digested with proteolytic enzymes and analyzed on a C18 HPLC column after preliminary separation steps and pre-column derivatization with phenylisothiocyanate (PITC). To identify the crosslink, half of the purified samples was treated with γ-glutamylamine cyclotransferase (GACT), an enzyme which splits ε(γ-glutamyl)lysine with the liberation of an equimolar amount of lysine. The calculation was based on the area of the isodipeptide peak, on the increase of the Lys peak after GACT treatment correlated with the change in isotope distribution and the recovery of standard ^{3}H-Glu-Lys what was added to the samples as a tracer at the beginning of the process.

Figure 13 shows the elution profile of the sample without (A) and after GACT treatment (B) on a C18 HPLC column. The injected amount was 1.0 mg of the original protein. The Glu-Lys peak (the retention time of the isodipeptide and of Lys

was continuously established with pure standards) corresponds to 640 pmol. The radioactivity of the fractions containing ^{3}H-Glu-Lys and Lys was also demonstrated.

The results from this study demonstrates the presence of ε(γ-glutamyl)lysine (Glu-Lys) crosslinks in filarial preparations. Therefore, the filarial inhibition and toxicity demonstrated with transglutaminase inhibitors in part is a function of direct interference with the formation of these biochemical crosslinks.

EXAMPLE VI
Effect of Cystamine, Iodoacetamide and MDC on Release of Microfilaria from *Brugia malayi*

The present example demonstrates the microfilaricidal and microfilaricidal activity of the particular TGase inhibitors, iodoacetamide, cystamine and MDC. These particular transglutaminase inhibitors are examples of active site inhibitors (cystamine and iodoacetamide) and competitive inhibitors (MDC) of transglutaminase activity. The effects of these agents on microfilarial release and larval stage (L_3) viability was determined.

Adult worms of *B. malayi* were prepared as described *supra* (see Example IB). After a 24-hour incubation period with the indicated concentrations of cystamine (50 μM, 100 μM, 200 μM, 400 μM, 600 μM and 800 μM), the parasites were removed and 10 μl of spent medium was examined under the light microscope to count the microfilariae.

Table XIV
***In Vitro* effect of Cystamine (Active site inhibitor) on *Brugia malayi* infective larval stage (L3) viability**

Cystamine RPMI 1640+FSC	L3 viability (hours after incubation)			
	1	2	4	24
Controls	+++	+++	+++	+++
5 mM	+++	+++	++	-
1 mM	+++	+++	+++	-
800 μM	+++	+++	+++	+++
600 μM	+++	+++	+++	+++

Grading: -, Inactive; +, less active; ++, active; +++, very active

Twenty L3 were used for each concentration. Incubations at 37°C in CO_2 incubator.

As demonstrated at Table XIV, all tested concentrations of iodoacetamide greater than 0.3 μM were effective in inhibiting Mf release by at least 60%, with concentrations of 600 μM or greater providing about 100% inhibition, compared to untreated controls.

TABLE XV
In vitro macro filaricidal and microfilaristatic activity of cystamine

Cystamine RPMI 1640+FCS						
0(Cont)	+++	+++	+++	+++	+++	3,500
800 μM	+++	+++	-	ND	-	0 (100)
600 μM	+++	+++	-	ND	-	0 (100)
400 μM	+++	+++	+++	+	-	0 (100)
200 μM	+++	+++	+++	+++	+++	500 (86)
100 μM (49)	+++	+++	+++	+++	+++	1800
50 μM (40)	+++	+++	+++	+++	+++	2100

Grading: -, Inactive; +, less active; ++; active +++, very active
Two female worms were used for each concentration. Incubation at 37°C in CO_2 incubator.
ND - Not determined

Cystamine elicited significant inhibition of Mf release. 40% inhibition was demonstrated at dose levels of 50 μM, with 100% inhibition demonstrated at treatment doses of 400 μM and greater. The percent inhibition of Mf release was dose-dependent with both iodoacetamide and cystamine.

As demonstrated in Table XV, cystamine was also filaricidal to *B. malayi* infective larval stage (L_3) after a 24 h incubation in a 1 mM concentration of cystamine.

Table XVI
In Vitro **Macrofilaricidal and Microfilaristatic Activity of Irreversible TGase Inhibitors**

release inhibition)	Adult worm viability (hours after incubation) 1	2	4	16	24	Mf 24 h (%
Iodoacetamide						
0(cont)				+++	+++	3,900
5 μM				-	-	0 (100)
2.5 μM				+	-	0 (100)
1.25 μM				+	+	0 (100)
0.6 μM				++	+	162 (96)
0.3 μM (61)				+++	+++	1,500

As shown in Table XV, iodoacetamide was filaricidal to *B. malayi* infective larval stage (L_3) after a 24 h incubation in a 2.5 μM concentration of iodoacetamine. All lower concentrations of iodoacetamide tested were not filaricidal at the incubation times examined. A 5 μM, 16 hour preincubation with iodoacetamide was also demonstrated to be filaricidal to *B. malayi* infective larval stage (L_3).

L_3 viability was also examined after MDC or DDC incubation. DDC did not affect the viability of L_3 *B. malayi* at any of the concentrations tested. In contrast, concentrations of MDC of 200 μM after a 24 hour incubation were demonstrated to reduce L_3 viability 51% compared to non-treated controls. Higher concentrations of MDC (300 μM and 500 μM) reduced L_3 viability after only a 4 h incubation period. 4 hr incubation of L_3 *B. malayi* in 500 μM MDC reduced

viability 100%. See Table XVII.

Table XVI
Effect of MDC (Competitive inhibitor), DDC (Inactive analogue of MDC) on *Brugia malayi* L3 viability *in vitro*

Inhibitor/conc.	Hrs post-incubation Larvicidal Activity in %			
MDC	1 hr	4 hr	16 hr	24 hr
500 μM	None	100	100	100
300 μM	None	28	100	100
200 μM	None	None	None	51
100 μM	None	None	None	None
Controls	None	None	None	None
DDC				
500 μM	None	None	None	None
300 μM	None	None	None	None
200 μM	None	None	None	None
Controls	None	None	None	None

Experiments were conducted twice in duplicate. Twenty L3 were incubated per concentration. Incubations were at 37°C for 24 hours.

These results demonstrate that a broad scope of transglutaminase inhibitors are effective for providing the filarial inhibition and filaricidal activity described in the present inventive methods and agents.

The foregoing description of the invention has been directed to particular preferred embodiments in accordance with the requirements of the patent statutes and for purposes of explanation and illustration. It will become apparent to

those of skill in the art that modifications and changes in the appended claims may be made without departing from the scope and the spirit of the invention.

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CLAIMS:

1. A method for inhibiting maturation of and microfilariae production by female filarial nematodes comprising administering a transglutaminase inhibiting agent to the female filarial nematode.

2. The method of claim 1 wherein the transglutaminase inhibiting agent comprises a transglutaminase inhibitor selected from the group consisting of monodansyl cadaverine, putrescine, histamine, methyl amine, cystamine iodoacetamide and ß-phenyl propionylthiocholine.

3. A method for inhibiting maturation of and microfilarial production by *Brugia malayi* comprising treating a host female *Brugia malayi* nematode with a transglutaminase inhibitor in an amount sufficient to block said maturation and production.

4. The method of claim 1 wherein the filarial nematode is *Brugia malayi, Brugia pahangi, Brugia patei,* or *Brugia timori.*

5. The method of claim 1 wherein the filarial nematode is *Wuchereria bancrofti.*

6. The method of claim 1 wherein the filarial nematode is *Loa loa.*

7. The method of claim 1 wherein the filarial nematode is *Onchocerca volvulus.*

8. The method of claim 1 or 3 wherein the transglutaminase inhibitor is monodansyl cadaverine.

9. A method for inhibiting *in vitro* maturation of and microfilarial production by a *Brugia malayi*, *Brugia pahangi* or *Brugia patei* nematode comprising treating a female *Brugia malayi*, *Brugia pahangi* or *Brugia patei* nematode with a transglutaminase inhibitor in an amount sufficient to inhibit transglutaminase activity of said nematode.

10. The method of claim 9 wherein the female filarial nematode is *Brugia malayi*.

11. The method of claim 9 where the transglutaminase inhibitor is monodansyl cadaverine, putrescine, histamine methyl amine, cystamine, iodoacetamine, or ß-phenyl propionylthiocholine.

12. The method of claim 9 where the transglutaminase inhibitor is monodansyl cadaverine.

13. The method of claim 2 wherein the adult female filarial nematode is treated with transglutaminase inhibitor at about a 200 μM concentration.

14. The method of claim 3 wherein the amount of transglutaminase inhibitor results in about a 200 μM concentration in the host.

15. The method of claim 9 wherein the adult female nematode is treated with transglutaminase inhibitor at about a 200 μM concentration.

16. A method for killing filarial nematode comprising treating said nematodes with a filaricidal concentration of a transglutaminase inhibitor.

17. The method of claim 16 wherein the filarial nematodes are *Brugia malayi*, *Brugia pahangi*, *Brugia patei* or *Brugia timori*.

18. The method of claim 16 wherein the filarial nematodes are *Wuchereria bancrofti*.

19. The method of claim 16 wherein the filarial nematodes are *Loa loa*.

20. The method of claim 16 wherein the filarial nematodes are *Onchocerca volvulus*.

21. The method of claim 16 wherein the transglutaminase inhibitor is monodansyl cadaverine, putrescine, histamine methyl amine, or ß-phenyl propionylthiocholine.

22. The method of claim 16 wherein the transglutaminase inhibition is monodansyl cadaverine.

23. The method of claim 16 wherein the toxic concentration is

greater than about 300 μM.

24. A method for inhibiting maturation of and microfilarial production by a *Brugia malayi* female nematode existing within a mammalian host comprising treating said host with a transglutaminase inhibitor in an amount sufficient to inhibit maturation of and microfilarial production by a female *Brugia malayi* nematode.

25. The method of claim 24 wherein the transglutaminase inhibitor is monodansyl cadaverine, putrescine, histamine, methyl amine or ß-phenyl propionylthiocholine.

26. The method of claim 24 wherein the transglutaminase inhibitor is monodansyl cadaverine.

27. The method of claim 24 wherein the mammalian host is a rodent.

28. The method of claim 24 wherein the mammalian host is a human.

29. A method for killing an adult filarial nematodes existing within a mammalian host comprising treating said host with a filaricidal concentration of a transglutaminase inhibitor in a pharmacologically acceptable diluent.

30. The method of claim 29 wherein the transglutaminase inhibitor is monodansyl cadaverine or ß-phenyl

propionylthiocholine.

31. The method of claim 26 wherein the adult filarial nematode is treated with transglutaminase inhibitor at a greater than about 300 μM concentration.

32. The method of claim 26 wherein the mammalian host is a rodent.

33. The method of claim 26 wherein the mammalian host is a human.

34. A method for treating filariasis in a mammalian host comprising administering to a host a filaricidal concentration of a transglutaminase inhibitor.

35. A method for treating lymphatic filariasis in a mammalian host comprising administering to the host a transglutaminase inhibitor in an amount sufficient to kill adult filarial nematodes producing lymphatic filariasis.

36. A method for treating lymphatic filariasis produced by *Brugia malayi* in a mammalian host comprising administering to the host a transglutaminase inhibitor in an amount sufficient to kill adult *Brugia malayi* nematodes producing the lymphatic filariasis.

37. The method of claim 31, 32 or 33 wherein the transglutaminase inhibitor is administered parenterally.

38. The method of claim 34 wherein the transglutaminase inhibitor is administered intravascularly.

39. The method of claim 36 wherein the transglutaminase inhibitor is administered intravenously.

40. The method of claim 36 wherein the transglutaminase inhibitor is administered transdermally.

41. The method of claim 33, 34 or 35 wherein the transglutaminase inhibitor is administered internally.

42. The method of claim 34 wherein the adult filarial nematode producing the lymphatic filariasis is *Brugia pahangi*, *Brugia patei*, *Brugia timori* or *Wuchereria bancrofti*.

43. The method of claim 33, 34 or 35 wherein the transglutaminase inhibitor is monodansyl cadaverine.

44. The method of claim 33, 34 or 35 wherein the mammalian host is a human.

45. The method of claim 33 wherein the adult filarial nematode producing the filariasis is *Onchocerca volvulus* or *Loa loa*.

46. A transglutaminase inhibitor for use in the treatment of

filariasis.

47. A transglutaminase inhibitor for use in the killing of adult filarial nematodes.

48. A transglutaminase inhibitor for use in inhibiting production of microfilariae in a host infected with filarial nematodes.

49. The transglutaminase inhibitor of claim 46, 47 or 48 wherein the transglutaminase inhibitor is a reversible transglutaminase inhibitor or an irreversible transglutaminase inhibitor.

50. The transglutaminase inhibitor of claim 46, 47 or 48 defined further as selected from the group consisting of monodansyl cadaverine, dansyl cadaverine, putrescine, histamine, methyl amine, cystamine, iodoacetamide, ß-phenyl propionylthiocholine, amantadine, rimantidine.

51. The transglutaminase inhibitor of claim 46, 47 or 48 defined further as selected from the group consisting of histamine, putrescine, cystamine, monodansyl cadaverine, methyl amine, iodoacetamide, ß-phenyl propionylthiocholine, a 3,5 substituted 4,5-dihydroisoxazole, amantadine and rimantadine.

52. The transglutaminase inhibitor of claim 46 or 47 wherein the transglutaminase inhibitor is monodansyl cadaverine or ß-phenyl propionylthiocholine.

53. The transglutaminase inhibitor of claim 46 or 47 wherein the transglutaminase inhibitor is monodansyl cadaverine.

54. The transglutaminase inhibitor of claim 46 or 47 wherein the transglutaminase inhibitor is ß-phenyl propionylthiocholine.

55. The transglutaminase inhibitor of claim 46, 47 or 48 further defined as a reversible transglutaminase inhibitor selected from the group consisting of monodansylcadaverine, dansylcadaverine, putrescine, methyl amine, amantadine, histamine and rimantidine.

56. The transglutaminase inhibitor of claim 49 defined further as a reversible transglutaminase inhibitor selected from the group consisting of monodansylcadaverine, dansylcadaverine, putrescine, methyl amine, histamine, amantadine, rimantidine, and ß-phenyl propionylthiocholine.

57. The transglutaminase inhibitor of claim 49 defined further as a reversible transglutaminase inhibitor selected from the group consisting of monodansyl cadaverine, putrescine and histamine.

58. The transglutaminase inhibitor of claim 49 defined further as the reversible transglutaminase inhibitor monodansyl cadaverine.

59. The transglutaminase inhibitor of claim 49 defined further as an irreversible transglutaminase inhibitor selected from the group consisting of cystamine and iodoacetamide.

60. The transglutaminase inhibitor of claim 47 or 48 wherein the filarial nematodes are selected from the group consisting of *Onchocerca volvulus, Loa Loa, Brugia phangi, Brugia patei,*

Brugia timori, wuchereria bancrofti; and Brugia malayi.

61. The transglutaminase inhibitor of claim 47 or 48 wherein the filiarial nematode is *Brugia malayi, Brugia phangi* or *Brugia patei.*

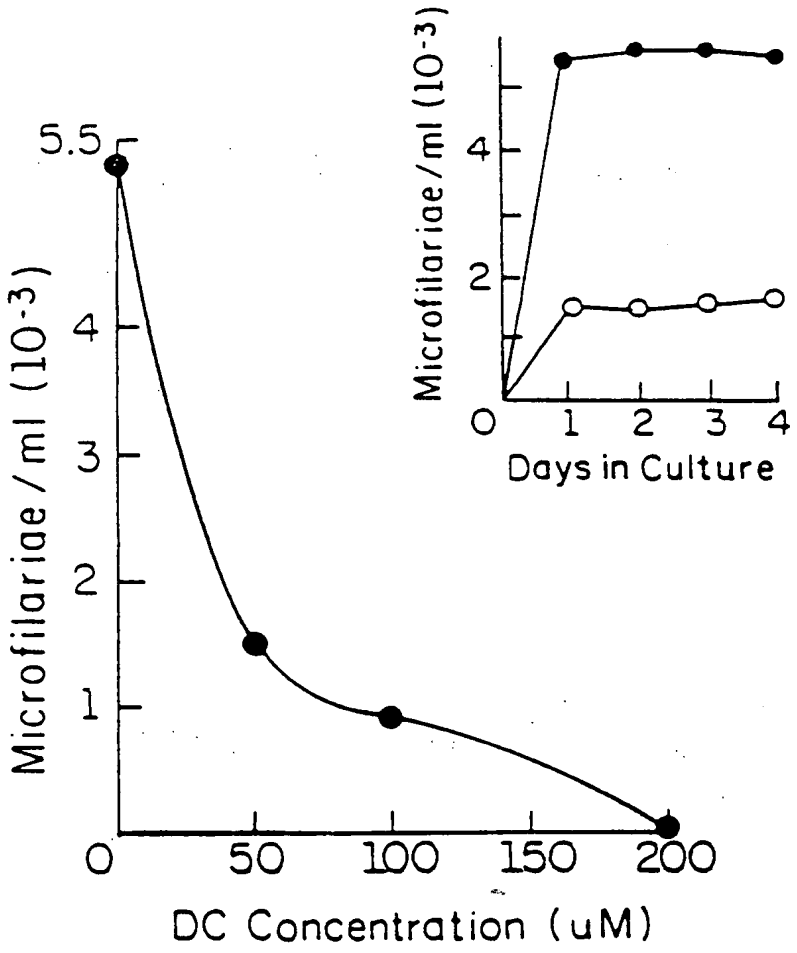

Microfilariae/ml (10-3)
5.5
4
3
2
1
0
50
100
150
200
DC Concentration (uM)
Microfilariae/ml (10-3)
4
2
0
1
2
3
4
Days in Culture

FIG. 1A

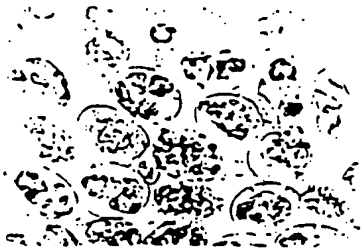


FIG. 1B

FIG 1C

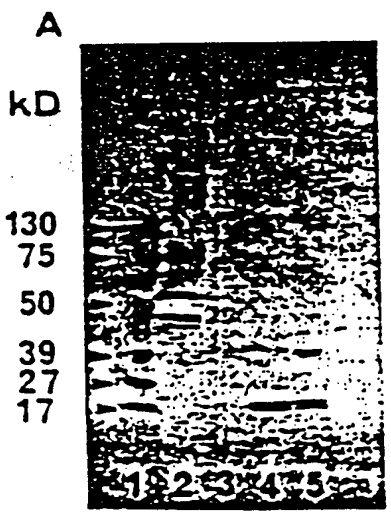


Fig. 2A

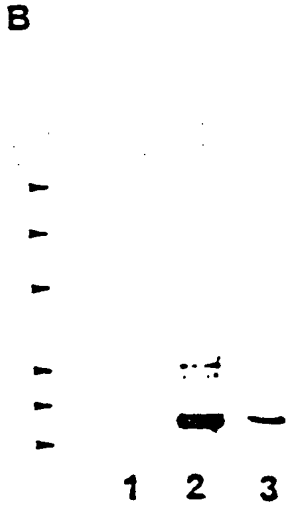


Fig. 2B

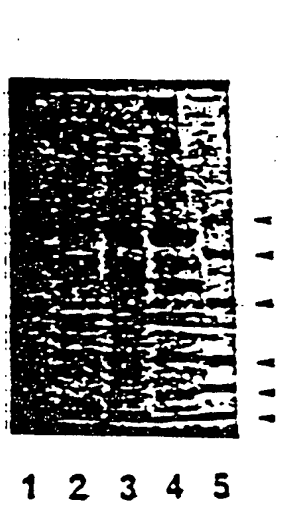


Fig. 2C

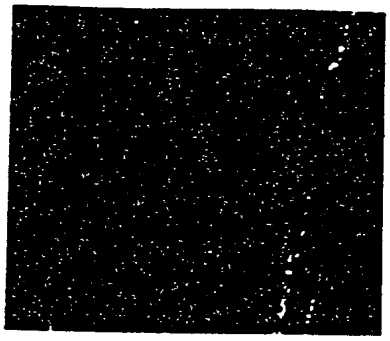


FIG. 3A

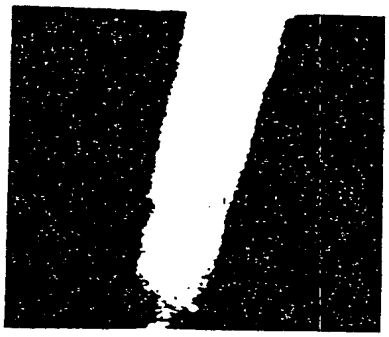


FIG. 3B

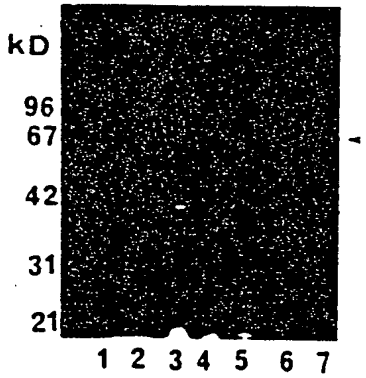


FIG. 4A

FIG. 4B

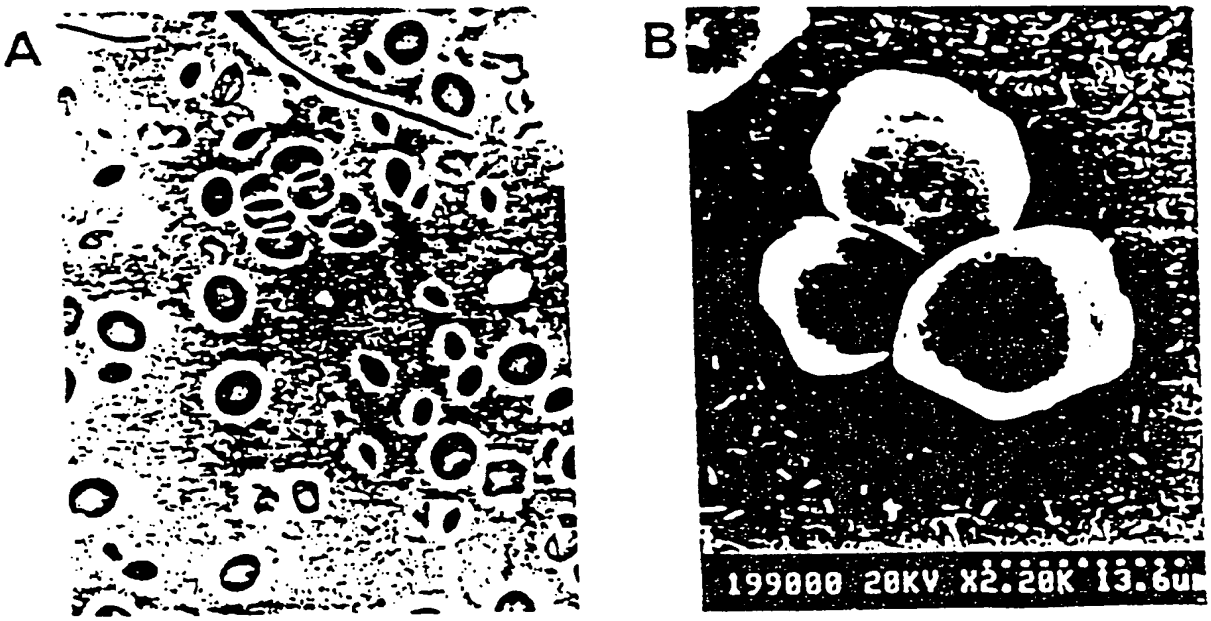


Fig. 5

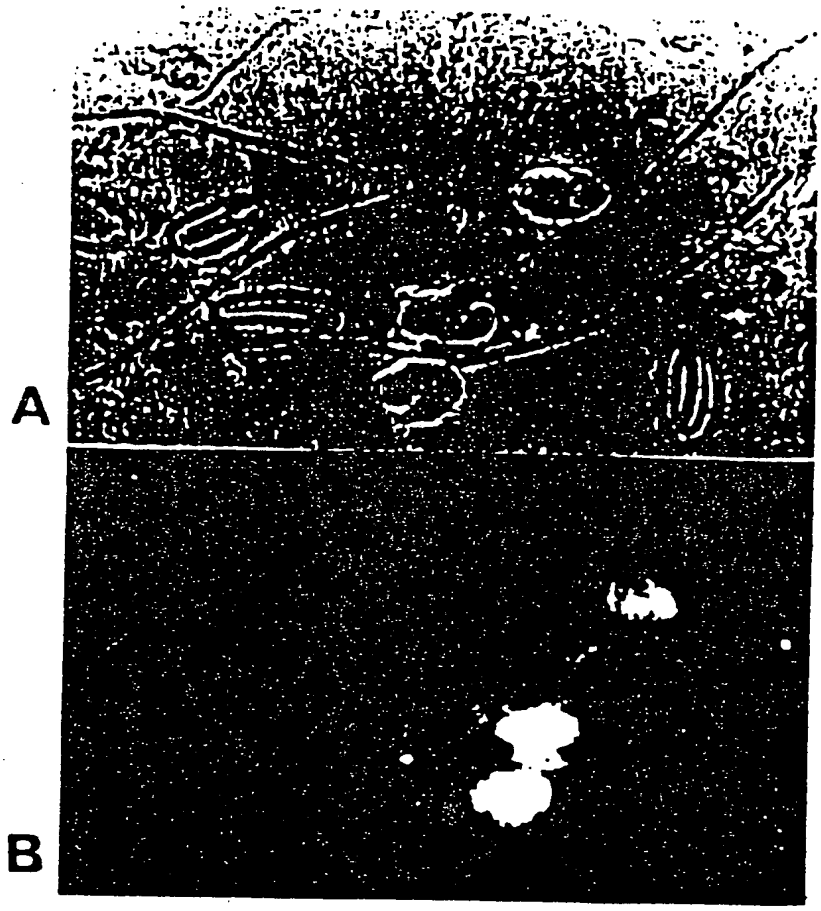


Fig. 6

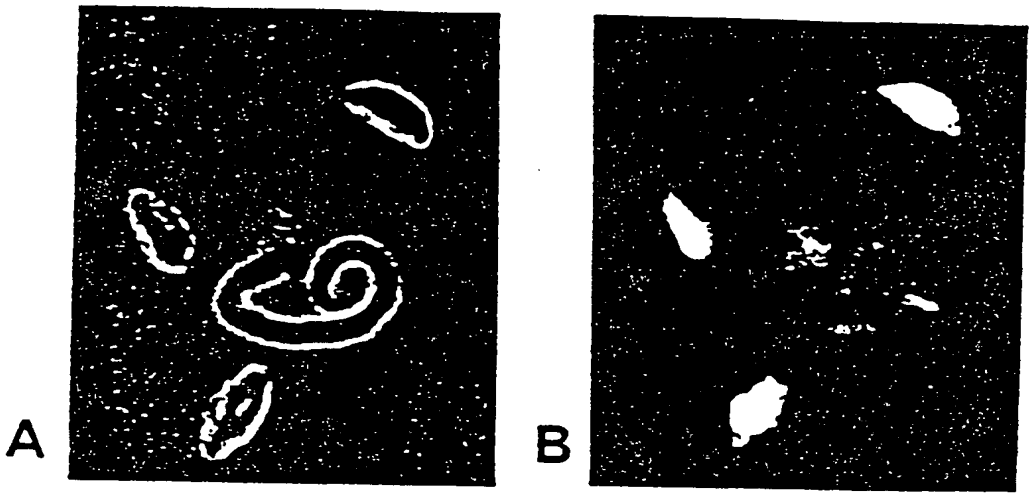
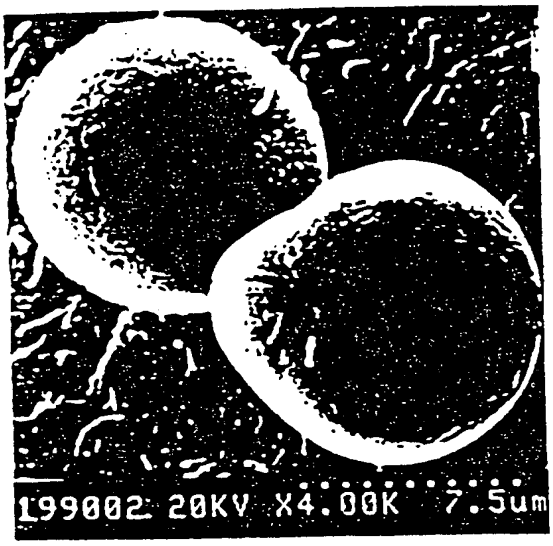


Fig. 7

A



B

Fig. 8

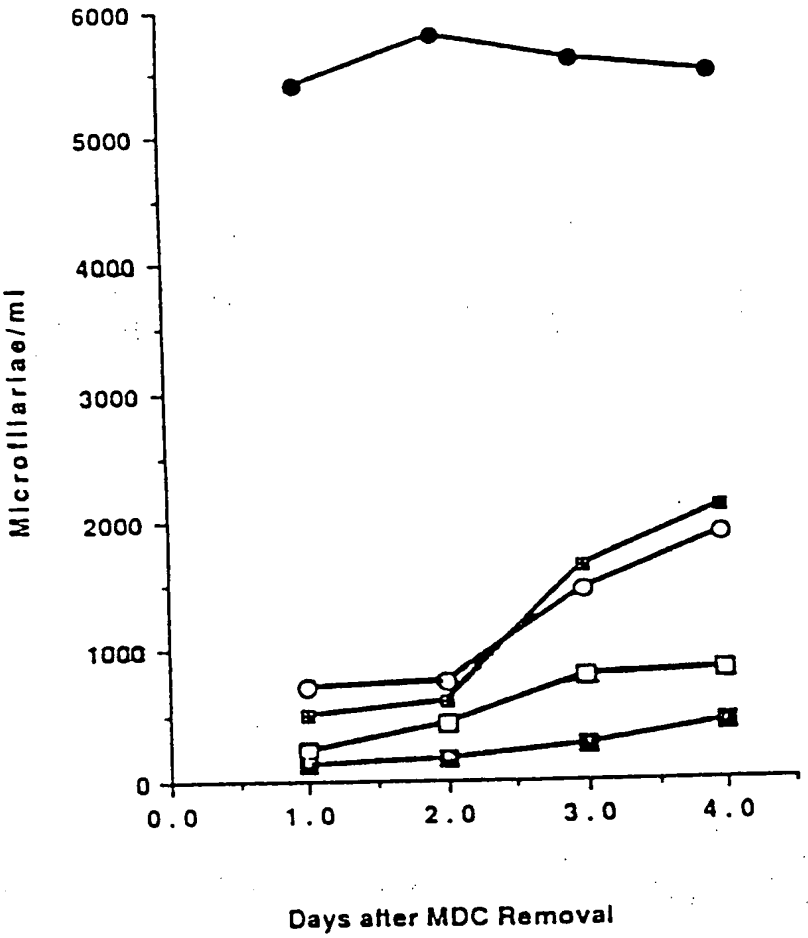

6000
5000
4000
3000
2000
1000
0
Microfilariae/ml
0.0
1.0
2.0
3.0
4.0
Days after MDC Removal

Fig. 9

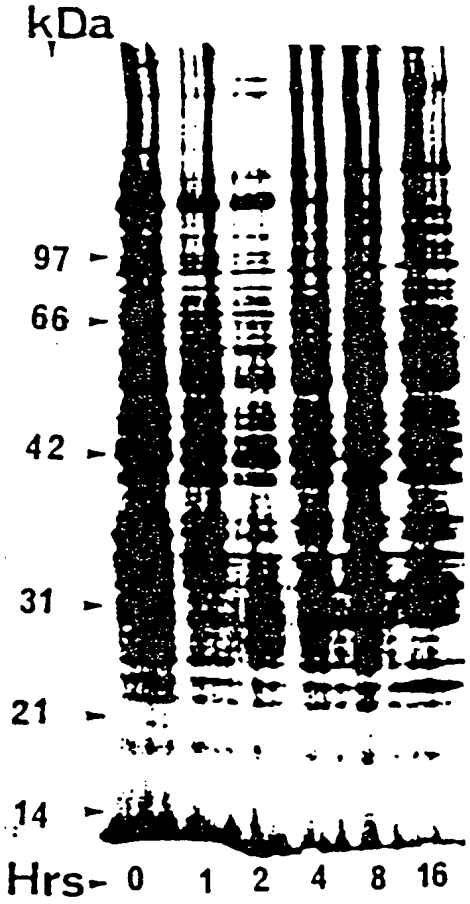


Fig. 10

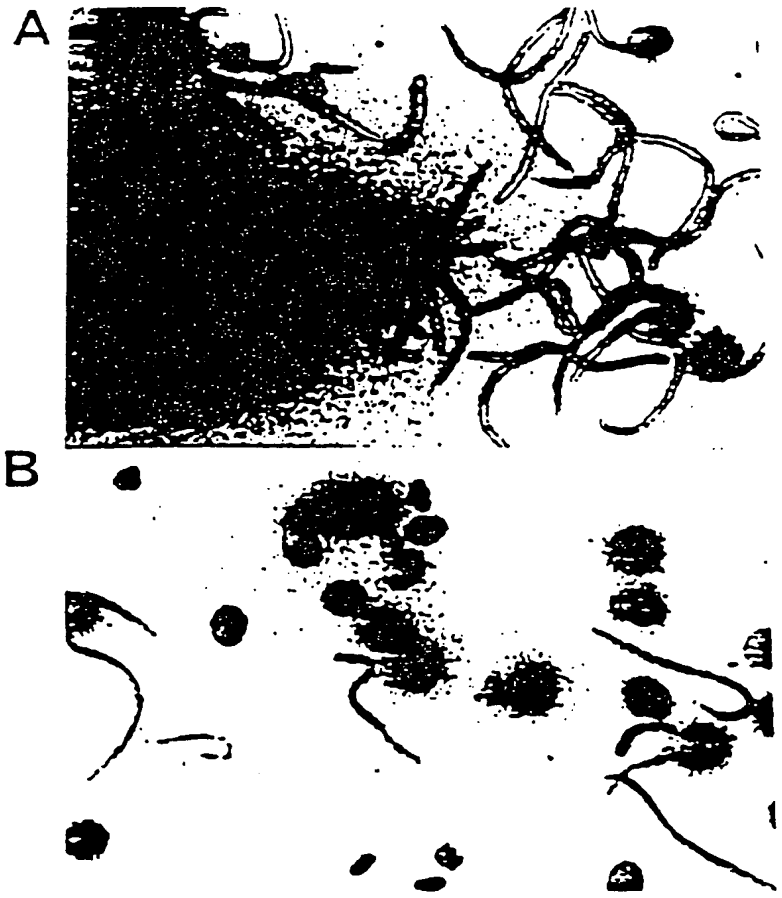
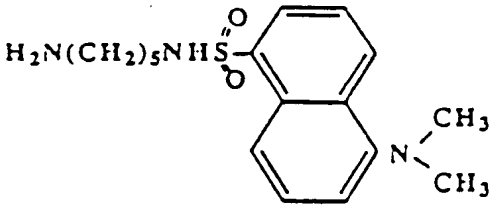
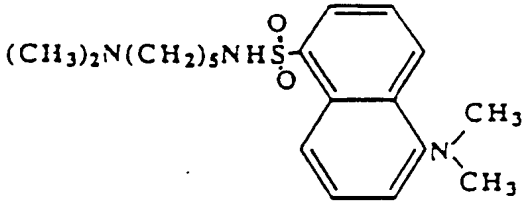


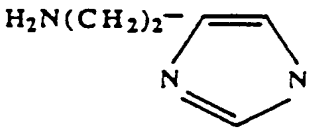
Fig. 11



Monodansylcadaverine



Dimethyldansylcadaverine



Histamine

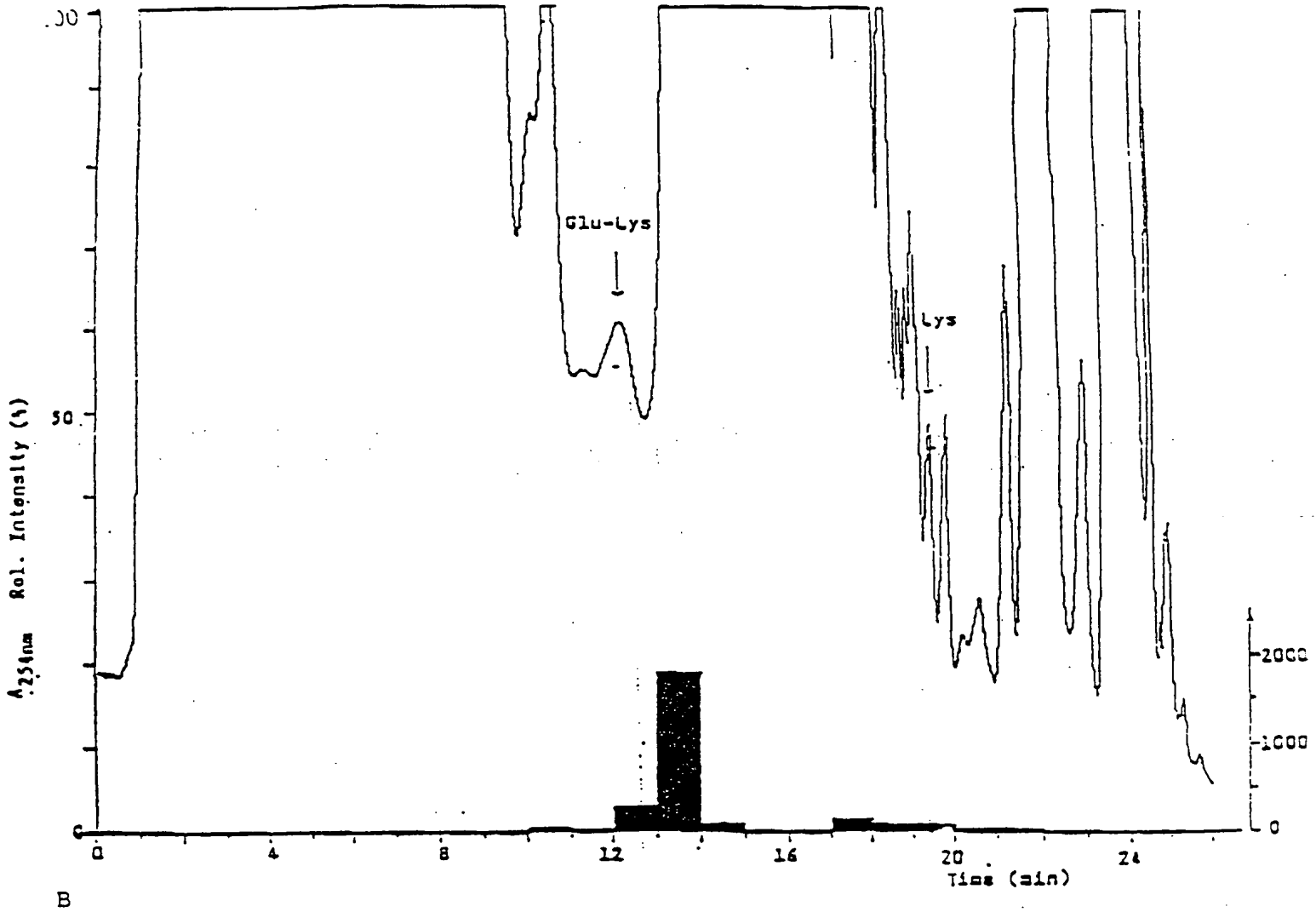
$NH_2(CH_2)_4NH_2$

Putrescine

CH_3NH_2

Methylamine

Fig. 12


100
50
0
Glu-Lys
Lys
Rel. Intensity (%)
2000
1000
0
0
4
8
12
16
20
24
Time (min)

B

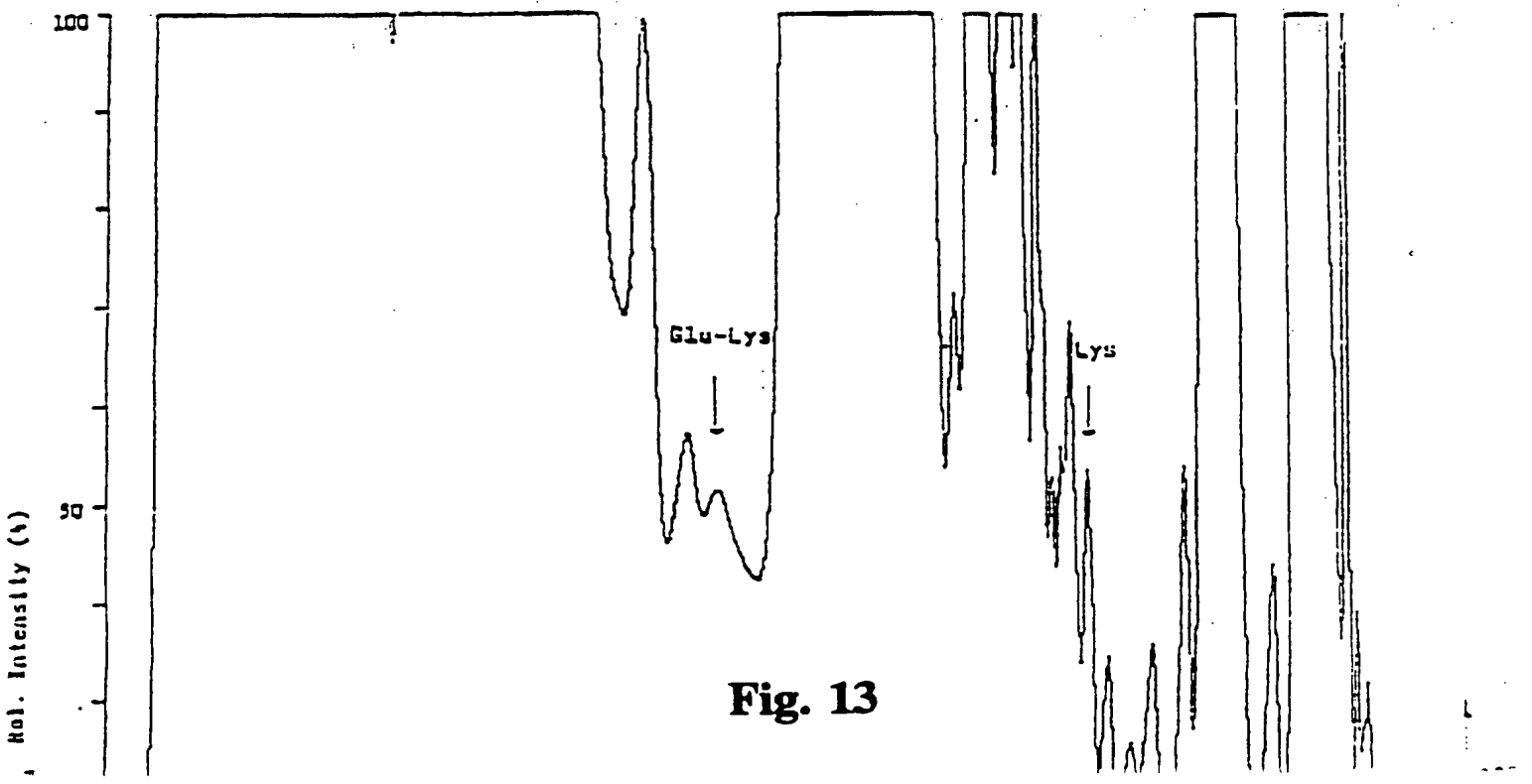

100
50
Glu-Lys
Lys
Rel. Intensity (%)

Fig. 13

(51) International Patent Classification 5 :
A61K 31/00, 31/13, 31/14. 31/16. 31/18
A61K 31/415

A3

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

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(54) Title: THE EFFECT OF TRANSGLUTAMINASE INHIBITION ON MICROFILARIAE DEVELOPMENT AND MACROFILARIAE VIABILITY

(57) Abstract

A method is described for inhibiting maturation and production of microfilariae in adult filarial nematodes. Inhibition of transglutaminase or transglutaminase mediated reactions utilizing a transglutaminase inhibitor to block the maturation and production of microfilaria by the adult organism, as well as kill the adult filarial organism. Experiments applying this method to several *Brugia* filarial infections, including *Brugia malayi*, were successful. Higher concentration of transglutaminase inhibitor prove the described filaricidal effect of the adult organism existing independent of a host or existing within a host organism.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07697

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 31/00, 31/13, 31/14, 31/16, 31/18, 31/415

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	The Journal of Biological Chemistry, vol. 260, no. 27, 25 November 1985, (Baltimore, US), K.N. Lee et al.: "Development of selective inhibitors of transglutaminase", pages 14689-14694 see abstract --	46-61
X	Biochemistry, vol. 17, no. 13, 1978, American Chemical Society, (Easton, US), G.E. Siefring, Jr. et al.: "Enzymatic basis for the Ca^{2+}-induced cross-linking of membrane proteins in intact human erythrocytes", pages 2598-2604 see page 2600, column 2, --	46-61

./.

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1st July 1991	07 AUG 1991
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MISS I. TAZELAAR

International Application No PCT/US 90/07697

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	H.J. Roth et al.: "Pharmazeutische Chemie III: Arzneistoffe", 1988, Georg Thieme Verlag, (Stuttgart, DE), page 161 see paragraph 6.2: Cycloalkylamine --	46-61
X	International Journal of Developmental Neuroscience, vol. 4, no. 5, 1986, Pergamon Journals Ltd, (Oxford, GB), G.M. Gilad et al.: "Cytotoxic effects of monodansylcadaverine and methylamine in primary cultures of rat cerebellar neurons", pages 401-405 see abstract --	46-61
X	Z. Parasitol., vol. 57, no. 2, 1978, Springer-Verlag, (Berlin, DE), M.M. Goil et al.: "Studies on sorbitol dehydrogenase from the parasitic nematode larvae of Phocanema decipiens", pages 117-120 see abstract; table 1 --	46-61
X	Molecular and Biochemical Parasitology, vol. 38, no. 1, 1 January 1990, Elsevier Science Publishers B.V., (Amsterdam, NL), R.-M. Wittich et al.: "Putrescine N-acetyltransferase in Onchocerca volvulus and Ascaris suum, an enzyme which is involved in polyamine degradation and release of N-acetyl-putrescine", pages 13-17 see page 14, column 1 --	46-61
X, P	Biochemical and Biophysical Research Communications, vol. 173, no. 3, 31 December 1990, Academic Press, Inc., (Duluth, US), K. Mehta et al.: "Significance of transglutaminase-catalyzed reactions in growth and development of filarial parasite, Brugia malayi", pages 1051-1057 see the whole article	46-61

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-45 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT-Rule 39.1. (iv) methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods

2. [X] Claim numbers *, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

* Claims searched completely: 50-59
* Claims searched partially: 46-49,60-61

A compound cannot be defined by its pharmacological activity

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

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2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.